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CLOZAPINE AND HALOPERIDOL INCREASE THE EXPRESSION OF PED/PEA-15: A PUTATIVE NOVEL MECHANISM FOR ANTIPSYCHOTIC-RELATED DIABETES

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ABSTRACT

Schizophrenia is a serious disorder that affects 1% of the population in the United States and Europe, and is associated with a significant reduction in life expectancy that approaches 20% compared with that of the general population. Current management of patients with schizophrenia involves the increasing use of atypical antipsychotic agents, such as risperidone, quetiapine, olanzapine, and clozapine. The use of these agents has been associated with increased morbidity from obesity, hyperlipidemia, development of new-onset diabetes, and, in rare instances, development of diabetic ketoacidosis. The precise mechanism for the abnormalities in carbohydrate and lipid metabolism is incompletely understood.. We investigated whether haloperidol and clozapine, respectively a typical and an atypical antipsychotics, directly affect insulin action in cultured cell models and *in vivo*. In our experimental paradigms, both haloperidol and clozapine reduced insulin-stimulated glucose uptake in a time- and concentration dependent manner, although with a different efficacy. Indeed, pre-treatment with clozapine, but not with haloperidol, prevented insulin effect on insulin receptor (IR) and IR substrate-1/2 (IRS-1/2) tyrosine phosphorylation.

Moreover, both drugs reduced insulin-dependent phosphorylation of protein kinase C-ζ (PKC- ζ), and induced an insulin-independent increase of phosphorylated Akt. These effects were paralleled by increased expression of Ped/Pea-15, an Akt substrate and inducer of insulin resistance. Similar changes of these signalling proteins were detected

in caudate-putamen and in skeletal muscle of mice treated with either haloperidol or clozapine. Thus, antipsychotics may impair insulin action, at least in part, by up-regulating Ped/Pea-15 and inhibiting activation of PKC- ζ.

INTRODUCTION

Schizophrenia, Type 2 Diabetes Mellitus and Antipsychotics

Schizophrenia is a major highly debilitating psychiatric disorder with a worldwide prevalence of about 1% ([1]). Among schizophrenic patients life expectancy is 20% shorter than in general population ([2]). This is accounted for, at least in part, by circulatory, respiratory and metabolic illnesses ([3; 4; 5; 6]). Several lines of evidence, replicated over many decades, have indicated that schizophrenic patients have an higher prevalence of impaired glucose tolerance, insulin resistance and type 2 diabetes mellitus than general population ([7; 8; 9]). A family history of type 2 diabetes mellitus is found in 18-19% of schizophrenic patients as compared to 1,2-6,3 % in the general population ([10; 11]). Although the patho-physiological bases of this relationship are not clearly understood, a combination of genetic and environmental factors, including lifestyle and medications, is likely to be involved in the dysregulation of glucose metabolism observed in these patients ([12; 13; 14]). Indeed, the treatment with antipsychotics has been associated with weight gain, impaired glucose tolerance, insulin resistance and type 2 diabetes mellitus ([15; 16]).

On the other hand, several reports although not all, suggestthat schizophrenia is associated with abnormal glucose metabolism independently of antipsychotic use. A few studies have investigated that diabetes has an increased prevalence in the families of schizophrenia probands. Mukherjee et al. (1989) described an increased prevalence

of type 2 diabetes mellitus (T2DM) among first-degree relatives of schizophrenia patients.

One study (Spelman et al., 2007) found an increased prevalence of impaired glucose tolerance in an oral glucose tolerance test (GTT) in newly diagnosed, antipsychotic-naïve patients with schizophrenia (10.8%) and their first-degree relatives (18%) compared to healthy controls (0%). However, the relatives and healthy controls were not well matched for age, body mass index (BMI), and smoking habit, which are known risk factors for developing diabetes.

In summary, there are more considerable evidences that the prevalence of diabetes is increased in patients with schizophrenia and is linked to the use of antipsychotic medications. The underlying mechanism for the increased prevalence of type 2 diabetes in treated schizophrenic population is unknown. Several studies have failed to find a correlation between the duration of therapy and antipsychotic medications, and others have found significant differences between the older-generation antipsychotic medications and the newer atypical agents.

The studies are often confounded by concomitant weight gain and dyslipidemia, which are known diabetic risk factors. Increased abdominal obesity, especially visceral obesity, can increase insulin resistance and contribute to hyperglycemia and diabetes both in healthy subjects and patients with schizophrenia. As diabetes occurrence is not always associated with weight gain, monitoring weight alone may be insufficient to screen for DM risk. The methods that can be used to assess the effects of medications

on glucose and insulin metabolism include (ranked least to most sensitive/reliable): random glucose, glycated haemoglobin (HbA1C), fasting plasma glucose, homeostasis model assessment insulin resistance (HOMA-IR), post-prandial glucose, the oral glucose tolerance test (OGTT) and the intravenous glucose tolerance test (IVGTT), and the hyperinsulinaemic-euglycaemic clamp.

Even if an increased incidence of diabetes has been reported in schizophrenic patients before the introduction of the neuroleptics, and an association between glucose metabolism impairment and the use of first generation antipsychotic drugs (i.e. haloperidol) has been suggested ([17]), current evidence indicates that second generation antipsychotics, (or atypical antipsychotics) may induce hyperglycemia, exacerbation of existing diabetes or onset of diabetes mellitus ([18; 19; 20; 21]).

Clinical data indicate a significant variability in drug-induced abnormalities on glucose metabolism. Clozapine and olanzapine are associated with the greatest risk of developing type 2 diabetes mellitus.

Different mechanisms may be responsible for the metabolic side effects of anti-psychotics leading to weight gain and insulin resistance and ultimately to type 2 diabetes.

Carbohydrate and lipid homeostasis linked to energy conservation and expenditure is dependent on a complex interplay of factors that regulate food intake and metabolism. Glucose intolerance, as seen in patients receiving atypical antipsychotic medications, might be expected to be influenced by defects in insulin secretion and sensitivity,

abnormalities in the secretion of gut hormones that promote insulin secretion, secretion and release of insulin counter-regulatory hormones such as glucagon, or alterations in the regulation of food intake via central and peripheral mechanisms.

However, the molecular events underlying their actions are poorly understood. All antipsychotics share a common mechanism of dopamine D2 receptor occupancy, even with a different degree ([22; 23]). The D2 receptor family inhibits adenylyl cyclase in downstreaming transductional pathway. In addition to signaling through the second messenger 3'-5'- cyclic adenosine monophosphate (cAMP), activation of D2 receptor family stimulates the assembly of a complex containing β-arrestin2, protein phosphatase 2 A (PP2A) and Akt ([24]). Akt, also known as protein kinase B (PKB), is a serine-threonine kinase that has been largely studied for its role in growth factor-mediated cell survival, cell-cycle progression, and transcriptional regulation ([25]). Akt plays a pivotal role in the regulation of glucose metabolism. It is rapidly activated by insulin and, in skeletal muscle cells and adipocytes, it mediates the translocation of glucose transporter 4 (GLUT4) onto the plasma membrane ([26; 27; 28]). Moreover, Akt also participates in the complex mechanism involved in insulin desensitization ([29; 30; 31]) and protection from apoptosis ([32]). The molecular mechanisms elicited by Akt are only partially known ([33; 34]). Akt phosphorylates a wide variety of substrates, including the antiapoptotic protein Ped/Pea-15 ([35]).

Ped/Pea-15: a new potential target involved in antipsychotic-related diabetes

Phosphoprotein enriched in diabetes/phosphoprotein enriched in astrocytes (Ped/Pea-15) is a cytosolic phosphoprotein widely expressed in different tissues and highly conserved in mammals. It binds to and modulates the function of a number of signaling proteins and effectors. Ped/Pea-15 binds several pro- and anti-apoptotic proteins thereby exerting a broad anti-apoptotic function. It also controls mitogenic signaling by binding extracellular-regulated kinases (ERKs) and anchoring ERKs to the cytoplasm. Indeed, changes in Ped/Pea-15 expression play an important role in tumor development and sensitivity to anti-neoplastic agents. Ped/Pea-15 binds to phospholipase D, enhancing its stability and increasing intracellular diacylglycerol levels. This effect, in turn, activates classical protein kinase C isoforms and generates resistance to insulin action on glucose metabolism in peripheral tissues. Protein kinase C dysregulation by Ped/Pea-15 also impairs glucose-stimulated insulin secretion in β cells in mice.

Ped/Pea-15 gene maps on human chromosome 1q21–22 and is overexpressed in type 2 diabetics as well as in the euglycemic offspring from these individuals. Interestingly, in these same subjects, Ped/Pea-15 levels correlate with insulin resistance. Ped/Pea-15 cellular levels are regulated by ubiquitinylation and proteasomal degradation. However, run-on experiments in cultured cells from type 2 diabetic subjects demonstrated that, at least in part, the overexpression observed in these subjects is caused by transcriptional

abnormalities. The molecular details responsible for these abnormalities and the mechanisms responsible for Ped/Pea-15 gene regulation are still unclear.

Ped/Pea-15 is modulated by phosphorylation at Ser116, by calcium-calmodulin kinase (CaMKII) and Akt ([35]) and at Ser104 by PKC ([36; 37]) (Fig. 1). Ped/Pea-15 phosphorylation regulates its interaction with intracellular partners ([38]) and controls its proteasomal degradation ([39]). Increased expression of Ped/Pea-15 has been detected in patients with type 2 diabetes and their first degree relatives ([37; 40]). Moreover, transgenic mice overexpressing Ped/Pea-15 display abnormal glucose tolerance, insulin resistance and increased susceptibility to develop diabetes following weight gain ([41]). Overexpression of Ped/Pea-15 in cultured skeletal muscle cells inhibits insulin-induced activation of Protein kinase C- ζ (PKC- ζ), thereby impairing GLUT4 translocation and glucose uptake ([41; 42]).

We have previously characterized the brain expression of the Ped/Pea-15 mRNA by *in situ* hybridization (Fig. 2). This brain areas involved in the expression are the Hippocampus (Fig. 2A), mamillary bodies (Fig. 2A), Frontal cortex (Fig. 2B) and entorhinal cortex (Fig. 2B) (data not published).

Here we show, by *in vivo* and *in vitro* paradigms, that both haloperidol and clozapine increase Akt activity and Ped/Pea-15 protein expression in neurons and skeletal muscle cells. This is paralleled by decreased PKC- ζ activation and deranged insulin-stimulated glucose uptake.

MATERIALS AND METHODS

General. Media, sera and antibiotics for cell cultures were from Invitrogen Ltd. (Paisley, United Kingdom). Phospho-phospho-Ser473 Akt antibodies were purchased from Cell Signaling Technology (Beverly MA). Actin antibody was from Sigma (St. Louis, MO). Antibodies directed against phospho-PKC ζ /PKC ζ, Akt, were from Santa Cruz Biotechnology (Santa Cruz, Calif.). IRS1, IRS2 and Phospho-Tyr antibodies were from Upstate Cell Signaling Technology (Lake Placid, NY). Antibodies to Ped/pea-15 have been previously described ([37]). Electrophoresis and Western blot reagents were from Bio-Rad (Richmond, Va.). 2-deoxy-[^{14}C] glucose and ECL reagents were from GE Healthcare . Other reagents were from Sigma.

In vitro studies:

Cell culture procedures. PC12 transplantable rat pheochromocytoma cells and L6 cells were plated and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 5% horse serum (for PC12 cells), 100 μg/ml streptomycin, and 100 IU/ml penicillin at 37 C in a humidified atmosphere of 95% air and 5% CO2.

Drug preparation. Clozapine was a generous gift of Novartis Pharma (AG, Switzereland). Haloperidol was purchased by Sigma-Aldrich (Stheinheim, Germany). Both compounds were dissolved in Dimethyl sulfoxide (DMSO from Sigma Aldrich) at

the concentration needed, according to previously described procedures in similar experimental paradigms ([43; 44; 45; 46]).

2-deoxy-glucose (2-DG) uptake. 2-deoxyglucose (2-DG) uptake was measured as previously reported ([47]). Briefly, confluent cells were incubated in DMEM supplemented with 0,25 % albumin for 18 h at 37 C. The medium was aspirated and cells were further incubated for 30 min in glucose-free buffer (5 mM KCl, 120 mM NaCl, 1,2 mM MgSO4, 10 mM NaHCO3, 1,3 CaCl2, 1,2 mM KH2PO4, and 20 mM Hepes, pH 7.8, 2% bovin serum albumin). The cells were incubated in the same buffer supplemented with 100 nM insulin for 30 min, and incubated for further 10 min in glucose-free buffer containing 2-DG (final concentration 0,2 mM) and 0,5 μCi/assay [^{14}C]2-DG. The cells were finally lysed and 2-DG uptake was determined by liquid scintillation counting.

Western blot analysis and immunoprecipitation. Cell lysates were solubilized in lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 mM Na4P2O7, 2 mM sodium orthovanadate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4, and 1% Triton for 2 h at 4 C. Lysates were clarified by centrifugation at 12,000 x g for 15 min at 4 C, and aliquots were either immunoprecipitated with the indicated antibodies or directly separated by SDS-PAGE before analysis by Western blot, as previously described ([48; 49]).

In vivo studies:

Animal care. 3-months-old male C57/BL/KsJ mice were purchwwased from the Charles River Laboratories (Milan, Italy). Animal were housed under 12-h light/12-h dark cycle in a temperature and humidity controlled colony room and were given pelleted food and water *ad libitum* in a specific pathogen-free environment. All procedures were conducted in accordance with the NIH *Guide for Care and Use of Laboratory Animals* (NIH Publication N0.85-23, revised, 1985) and were approved by local Animal Care and Use Committee. Care was taken to minimize animal number and sufferance. Two hours after the last injection the mice were decapitated to obtain protein extracts from frontal cortex (FC) and from caudate-putamen (CP) using the rat brain atlas by Paxinos and Watson ([50]) as an anatomical reference (approximately from Bregma 6,70 mm to 4,20 mm and from Bregma 2,20 mm to -0,40 mm for FC and CP respectively).

Drug preparation and treatment. Clozapine and Haloperidol were dissolved in lactic acid (0.1% in NaCl), adjusted to pH 5.5 adding NaOH and administered intraperitoneally to the animals. The dose of haloperidol used in these studies was 0.8 mg/Kg for 21 days; clozapine was used at 10 mg/Kg for 21 days. The doses of both drugs were chosen according to previous studies showing a behavioural and transcriptional effect ([51; 52; 53; 54; 55]). The animals were sacrificed 90 min after the last injection.

Tissue collection and Immunoblotting. Tissue samples (frontal cortex, caudate putamen and quadriceps muscles) were rapidly collected, snap frozen in liquid nitrogen and stored at -80 C for subsequent Western blotting. Tissue samples were homogenized in a Polytron omogenizer (Brinkman Instruments, Westbury, NY) in T-PER TISSUE Protein Extraction Reagent buffer (25 mM bicine, 150 Mm sodium chloride pH 7.6, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, pH 7.4) (PIERCE Rockford, IL) according to the manufacturer's instruction. Total homogenates were centrifuged at 12,000 x g for 30 min at 4 C and were subjected to sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) with 10% gels. Proteins separated on the gels were electroblotted onto nitrocellulose filters as described above and membranes were probed with specific antibodies, as indicated.

Data Analysis A one factor analysis of variance (ANOVA) was used to analyze treatment effect. Any significant ANOVA were further analyzed by Student-Neuman-Keuls post hoc test to determine the specificity of the effect. p values < 0.05 were considered statistically significant.

RESULTS

Effect of haloperidol and clozapine on glucose uptake in cultured cells.

We have evaluated 2-deoxyglucose (2-DG) uptake in PC12 cells following the treatment with either 12.5 μM haloperidol or 1.5 μM clozapine for 24 h. No significant change of basal 2-DG uptake was induced by both compounds (Fig. 3A). However, both haloperidol and clozapine almost completely blunted insulin effect. Comparable results were obtained following identical treatment of L6 myotubes (Fig. 3B). Indeed, insulin elicited 2-DG uptake by 25% and 45% respectively, in PC12 and in L6 cells, while it failed to induce significant changes in the presence of either haloperidol or clozapine (Fig. 3A and 3B).

Effect of haloperidol and clozapine on insulin signalling in cultured cells.

In order to investigate at what level insulin action was impaired, L6 cells have been incubated with 12.5 μM haloperidol or 1.5 μM clozapine for 24 h and with insulin for additional 30 min. No changes of the cellular content of the insulin receptor (IR) and of the insulin receptor substrates, (IRS1 and IRS2) were decetcted by Western Blot with specific antibodies (Fig. 4A). Insulin exposure increased tyrosine phosphorylation of IR, IRS1 and IRS2 in control and in haloperidol treated cells (Fig. 4A). At variance, clozapine-treated cells displayed a drastic reduction of insulin-stimulated IR, IRS1 and IRS2 tyrosine phosphorylation (Fig. 4A).

We also investigated whether PKC- ζ and Akt, two major kinases involved in the regulation of glucose uptake, were affected by the antipsychotic compounds. Protein levels of PKC- ζ were similar in untreated cells and in cells exposed to either haloperidol or clozapine (Fig. 4B), while insulin-induced PKC- ζ phosphorylation at Thr 410 was reduced by both drugs. No change in basal PKC- ζ phosphorylation was detected, instead. Conversely Akt cellular content was slightly increased (difference was not statistically significant) by both haloperidol and clozapine.

Nevertheless, insulin-independent phosphorylation at Ser 473 (which represents an activation marker) was significantly increased by both compounds (p<0.05). In parallel with the early signalling steps, insulin effect on Akt phosphorylation was conserved upon haloperidol and reduced upon clozapine pre-treatment of the cells. Very consistent results were obtained in PC12 cells (data not shown).

Effect of haloperidol and clozapine on Ped/Pea-15 expression.

Next, we addressed whether haloperidol and clozapine may affect the cellular content of Ped/Pea- 15. Indeed, Ped/Pea-15 is an Akt substrate ([35]), whose overexpression causes insulin resistance in cellular and animal models ([37; 41]) and associates to insulin resistance in humans ([40]). Both haloperidol and clozapine treatment of PC12 (data not shown) and L6 cells (Fig. 4 B) for 24 h increased Ped/Pea-15 cellular levels. Despite a reduction in the absolute amount, raised Ped/Pea-15 content was also observed in the presence of cycloheximide (30 μM), a protein synthesis inhibitor, suggesting that, at

least in part, regulation occurred at post-translational level both in PC12 (Fig. 5A) and in L6 cells (Fig. 5B).

Therefore, PC12 and L6 cells were incubated with raising concentrations of haloperidol (1-100 μM) ([43; 44]) and clozapine (0.1-25 μM) ([43; 45]) for different time points (6, 12, 24 and 48 h), prior to measure the levels of phosphorylated Akt and Ped/Pea-15. The time- and concentration-dependent increase of Akt activation (Fig. 6A and 6C) closely paralleled that of Ped/Pea-15 cellular content, in PC12 cells (Fig. 6B and 6D), upon treatment with either haloperidol (Fig. 6A and 6B) and clozapine (Fig. 6C and 6D) ($p<0.001$).

Haloperidol treatment of L6 cells (Fig.6A) was less effective, as compared to PC12 cells, eliciting a significant Akt ($p<0.001$) activation as early as upon 12 h incubation with a concentration of 12.5 μM (Fig. 7A). Nonetheless, the regulatory effect on Ped/Pea-15 was consistently evident (Fig. 7B). Higher doses of haloperidol were tested but grossly impaired cell viability. Also, clozapine exposure led to parallel increases of cellular content of phosphorylated Akt (Fig. 7C) and of Ped/Pea-15 (Fig. 7D).

In vivo effects of haloperidol and clozapine on Akt, Ped/Pea-15, and PKC ζ

In order to verify whether antipsycothics may induce comparable changes *in vivo*, three groups of 8 C57/BL/KsJ mice were treated with haloperidol (0.8 mg/kg), clozapine (10 mg/kg) or vehicle.

Protein extracts from caudate-putamen (CP) and frontal cortex (FC) were obtained and assayed by Western blot with specific antibodies. Increased levels of phosphorylated Akt were detected in CP (Fig. 8A), but not in FC (Fig. 8B), in the animals treated either with haloperidol or with clozapine. This was paralleled by increased Ped/Pea-15 detection and reduced PKC- ζ phosphorylation in CP (Fig. 8A). A more marked decrease of phosphorylated PKC- ζ was observed upon clozapine treatment however (p<0.05). Again, no difference was detected in FC of the treated animals.

Similarly, quadriceps muscle specimens from haloperidol- and clozapine-treated mice featured increased phosphorylated Akt (Fig. 9A) and Ped/Pea-15 levels (Fig. 9B) as well as decreased PKC- ζ phosphorylation (Fig. 9C), compared to vehicle-treated controls.

DISCUSSION

The aim of the present study was to investigate the molecular mechanism by which haloperidol and clozapine may impair insulin sensitivity. Previous findings indicate that first and second generation antipsychotics directly worsen insulin-stimulated glucose transport ([43; 56]), thereby contributing to the abnormalities in glucose metabolism frequently observed in treated patients ([15; 19]). Indeed, glucose transport in insulin-sensitive tissues is rate-limiting for glucose metabolism, and impairments in this system may represent key abnormalities in inducing insulin resistance.

Our data show that exposure of PC12 and L6 cells, representative of neuron and skeletal muscle cells, respectively, to both haloperidol and clozapine caused an inhibition of insulin-stimulated glucose uptake. We have therefore evaluated whether different effectors of the insulin signal transduction cascade may be responsible for this impairment.

We showed that insulin receptor tyrosine auto-phosphorylation and kinase activity and insulin-stimulated Akt phosphorylation were differentially affected by typical and atypical antipsychotics being impaired in cells pre-treated with clozapine, while remaining unchanged after treatment with haloperidol. Thus, clozapine, but not haloperidol, impairs the early events of insulin signalling. On the other hand, common pathways were also affected by both antipsychotic drugs. Indeed, in the absence of insulin, phosphorylated Akt was increased following treatment with either haloperidol and clozapine. Moreover, insulin stimulation of PKC- ζ phosphorylation was drastically

impaired by both compounds, while protein content did not change. The increased Akt phosphorylation is consistent with previous observations suggesting that this protein represents a potential target of antipsychotic drugs ([44]). For example, it has been reported that clozapine activates Akt via phosphoinositide-3 kinase (PI3-K) signaling cascade ([45; 46; 57]). Other studies showed an increase of Akt phosphorylation by haloperidol ([58; 59; 60]). In addition, Akt has been envisioned as one of the potential schizophrenia susceptibility genes ([61; 62]).Alterations in Akt-GSK 3 α/β signalling likely contribute to the pathogenesis of schizophrenia ([60; 63]). Thus, one might speculate, that both haloperidol and clozapine may operate by enhancing Akt function, thereby counteracting the defect in schizophrenic patients ([58]).

However whether the increase of Akt phosphorylation induced by the antipsychotic compounds might also be involved in the impairment of insulin action on glucose uptake is less intuitive. Indeed, Akt is rapidly stimulated by insulin and mediates many insulin signals, including glucose uptake in different cellular systems ([64; 65]). In addition, insulin-stimulated Akt phosphorylation is reduced in adipocytes of type 2 diabetic patients and in skeletal muscle of impaired-glucose tolerant (IGT) individuals compared with control subjects ([66; 67; 68]), although these observations are not consistent in all the studies ([69; 70; 71]). On the other hand, Akt is also involved in insulin desensitization ([30; 31]), and increased Akt has been detected in several models of insulin resistance ([72]). Thus, it could be hypothesized that long term treatment with haloperidol and clozapine may induce persistent upregulation of Akt, which in turn may impair insulin signalling.

One of the potential mechanisms by which Akt may affect insulin signalling is the increase of Ped/Pea-15 protein stability ([35]). Indeed, Ped/Pea-15 is phosphorylated by Akt at serine 116 ([35]). This stabilizes the protein, likely preventing its degradation in the proteasomes ([35; 39]). Increased expression of Ped/Pea-15 has been associated to type 2 diabetes in humans ([37; 40]). Moreover, forced expression in mice and in cultured cells inhibits insulin action on glucose uptake by impairing PKC- ζ activation ([41; 42]). Defective activation of PKC-ζ has been also found in skeletal muscle biopsies taken during hyperinsulinaemic/euglycaemic clamp assay of obese and type 2 diabetic subjects ([68; 71]).

Ped/Pea-15 is also involved in the regulation of apoptotic cell death. Different experimental findings have suggested that overexpression of Ped/Pea-15 protects against stress-induced apoptosis ([35; 39; 72]). This antiapoptotic activity depends on phosphorylation of Ped/Pea-15 at Ser116. Thus, it is conceivable that elevated Ped/Pea-15 levels due to Akt activation, may contribute to the antiapoptotic action of antipsychotics in specific subsets of neurons. Indeed it has been suggested that antipsychotics may exhert a protective effect by up-regulation of Akt activity ([44; 45; 73]).

Raised Ped/Pea-15 levels may contribute to the potential neuroprotective effect, but also to the diabetogenic side effect of such compounds, impairing insulin action in peripheral tissues. Our data are consistent with the hypothesis that haloperidol and clozapine increase Akt activity, thereby causing hyperphosphorylation of Ped/Pea-15 at serine 116

(data not shown) and reducing its degradation. Intracellular accumulation of Ped/Pea-15 impairs activation of PKC- ζ and insulin-stimulated glucose uptake.

These observations are supported by the increased Akt phosphorylation and Ped/Pea-15 expression and decreased PKC- ζ phosphorylation found in the caudate-putamen of mice treated with either haloperidol or clozapine. This is a significant finding since the caudate putamen is a preferential target of D2-antagonists ([52; 75]). It is also intriguing that quadriceps muscles feature similar changes of the above signalling molecules and, together with data in the L6 cells, suggest that the skeletal muscle may represent a direct target, as well.

Moreover, it should be pointed out that clozapine, at variance with haloperidol, drastically decreases the early steps of insulin action, i.e. receptor and IRS phosphorylation. This is consistent with the higher impact exerted by several atypical antipsychotics on glucose tolerance ([76]). The mechanisms responsible for such alterations have not been identified, but they may also involve the increases of Akt, since, as previously reported, its hyper-activation impairs IRS1 function, and may affect receptor activity as well ([77; 78]). One might speculate that a different subcellular localization of Akt following exposure to clozapine may direct its function toward specific substrates. Alternative explanation could be searched in the different pharmacologic profile of the two antipsychotics (i.e. different affinity for and occupancy of dopamine receptors by clozapine compared to haloperidol).

In summary, our data suggest that first and second generation antipsychotics may contribute, with different degree and by acting at different level on transductional cascade of insulin, to the impairment of glucose metabolism. The molecular mechanism implicated in this effect involves intracellular effectors of the insulin signaling cascade. For instance, the increase of Akt activity enhances the levels of Ped/Pea- 15 by increasing its phosphorylation on Ser 116; as a consequence, Ped/Pea-15 inhibits the activation of atypical PKC- ζ and prevents insulin-stimulated glucose uptake.

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FIGURE LEGENDS

Figure 1. *Ped/Pea-15 protein function and structure.* Ped/Pea-15 is a ubiquitously expressed cytosolic protein which is modulated by phosphorylation at Ser116, by calcium-calmodulin kinase (CaMKII) and Akt and at Ser104 by PKC. Ped/Pea-15 gene maps on human chromosome 1q21–22. It binds to and modulates the function

of a number of signaling proteins and effectors and thus exerts its two mainly function in protection from apoptosis control of glucose transport.

Figure 2. *Ped/Pea-15 location in brain by In situ Hybridization.* We have detected the pattern of expression of Ped/Pea-15 by *in situ* hybridization using [^{35}S]-labeled antisense riboprobes. It is expressed in Hippocampus (A'), mamillary bodies (A''), Frontal cortex (B') and entorhinal cortex (B'').

Figure 3. *Antipsychotics inhibit insulin-stimulated glucose uptake in PC12 and L6 cells.* 2-deoxyglucose uptake was measured in PC12 (A) and L6 cells (B) after treatment for 24 h with haloperidol (12.5 μM) and clozapine (1.5 μM as indicated). Control cells received vehicle (DMSO) instead of

drugs. Cells were then exposed to 100 nM insulin as described in MATERIAL AND METHODS. Bars represent the means + SD of triplicate determination in four independent experiments. The significant differences (vs control), were determined by one factor analysis of variance (ANOVA). Positive samples were further analyzed by Student-Neuman-Keuls post hoc test to determine the specificity of the effect. p values < 0.001 were indicated by asterisks, ***.

Figure 4. *Effect of haloperidol and clozapine on insulin signaling in cultured cells.*

L6 cells have been incubated with haloperidol 12.5 μM and clozapine 1.5 μM for 24 hours and with insulin 100 nM for further 30 min. (A) Protein extracts were immunoprecipitated with anti IR (α- subunit), IRS-1 and IRS-2 antibodies, subjected to Western blotting (WB) with anti-phosphotyrosine, IR (β-subunit), IRS-1 and IRS-2 antibodies, as indicated. (B) Cell lysates were blotted with phospho-Ser- 473 Akt or phospho-Thr 410 PKCζ followed by reblotting with total Akt and PKCζ respectively and with Ped/Pea-15 antibodies. Equal loading of the samples was ensured by control blot with anti-actin antibododies. The blots were revealed by ECL

and autoradiography. The blots shown are representative of four independent experiments.

Figure 5. *Haloperidol and Clozapine increase Ped/Pea-15 expression.* The PC12 (A) and L6 (B) cells have been treated with haloperidol 12.5 μM and clozapine 1.5 μM for 24 h with or without cycloheximide 30μM (CHX), a protein synthesis inhibitor. Cells were lysed as described and lysates were blotted with anti Ped/Pea-15 antibody. Equal loading of the samples was ensured by control blot with anti-actin antibododies. The blots were revealed by ECL and autoradiography and subjected to densitometric analysis as described in Material and Methods. The blots shown are representative of four independent experiments. The significant differences (vs control), were determined by ANOVA. Student-Neuman-Keuls post hoc test was used to determine specificity of the effect. p values < 0.001 were indicated by asterisks, ***.

Figure 6. Effect *of antipsychotics on Ped/Pea-15 and Akt in PC12 cells.* PC12 cells were incubated for different times with increasing concentrations of haloperidol(A-B) and clozapine (C-D) as indicated. The cells were lysed as described, lysates were subjected to Western blotting

with phospho-Ser-473 Akt and Ped/Pea-15 antibodies and then reblotted with total Akt antibody. Equal loading of the samples was ensured by control blot with anti-actin antibododies. The blots were revealed by ECL and autoradiography. For phophorylated Akt, values were expressed as percent of P-Akt/Akt levels**,** for Ped/Pea-15 as arbitrary units derived from densitometric analysis,

further normalized on actin values, and shown with the bars**.** Data represent the means + S.D. from at least three experiments. ANOVA and Student-Neumans-Keuls post hoc test analysis revealed significant differences from the controls, *** $p<0.001$.

Figure 7. *Effect of antipsychotic drugs at on Ped/Pea-15 and Akt in L6 cells.* The L6 cells were incubated for different times with increasing concentrations of haloperidol (A-B) and clozapine(C-D) as indicated. The cells were lysed as described, subjected to Western blotting with phospho-Ser-473 Akt (pAkt) and Ped/Pea-15 and reblotting for total Akt. Equal loading of the samples was ensured by control blot with anti-actin antibododies. The blots were revealed by ECL and autoradiography. For phophorylated Akt, values were expressed as percent of P-Akt/Akt levels**,**

for Ped/Pea-15 as arbitrary units derived from densitometric analysis, further normalized on actin values, and shown with the bars**.** Data represent the means + S.D. from at least three experiments. ANOVA and Student-Neumans-Keuls post hoc test analysis revealed significant differences from the controls, *** $P<0.001$.

Figure 8. *Haloperidol and clozapine effect in frontal cortex and caudate putamen.*

Three groups of 8 mice were treated once a day for 21 days with single injection of saline, haloperidol (0.8 mg/kg) and clozapine (10 mg/kg). Phospho-Ser-473 Akt (pAkt), phospho-PKCζ and Ped/Pea-15 levels were measured in caudate putamen (A) frontal cortex (B), 30' after the last injection of drugs or saline. Equal loading of the samples was ensured by control blot with antiactin antibododies. Phosphorilated Akt and PKC ζ were expressed as percent of P-Akt/Akt and P-PKCζ/PKCζ levels**,**

Ped/Pea-15 as arbitrary units derived from densitometric analysis**.** Values were further normalized on actin values. Significant differences have been indicated by asterisks, *$p<0.05$, ***$p<0.001$.

Figure 9. *Haloperidol and clozapine effect in quadriceps muscle.* Mice were treated as described in fig.6 and sacrificed. Quadriceps were isolated and homogenated. Homogenates were then analyzed by WB as described in Fig.6. Equal loading of the samples was ensured by control blot with anti-actin antibododies. Phosphorilated Akt and PKCζ were expressed as percent of P-Akt/Akt and p-PKCζ/PKCζ levels**,** Ped/Pea-15 as arbitrary units derived from densitometric analysis**.** Values were further normalized on actin values. Significant differences have been indicated by asterisks, *p<0.05,**p< 0.01, ***p<0.001

PED/PEA-15 protein

Locus: 1q21-22
Protein MW: 15 KDa
Expression: Ubiquitous, mainly brain
Localization: mainly cytosol
Particular signs: DED, NES, serine phosphorilation sites
Known activity: protein-protein interaction

Binding proteins: ERK-1/2, FADD, FLICE, PLD, Akt

Proposed function:
• protection from apoptosis
• control of glucose transport

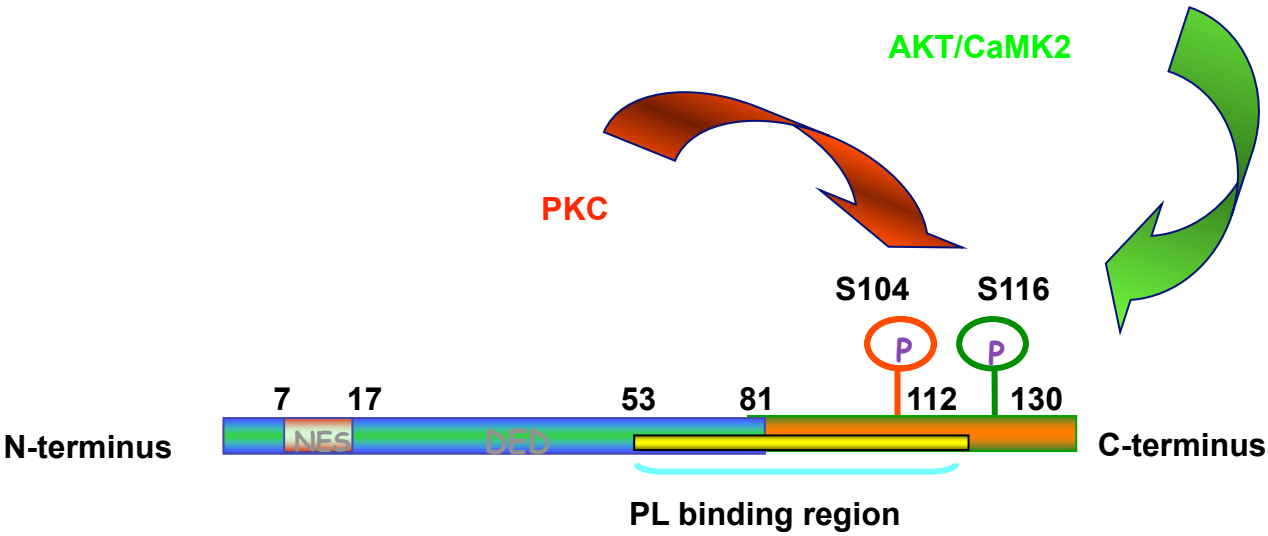
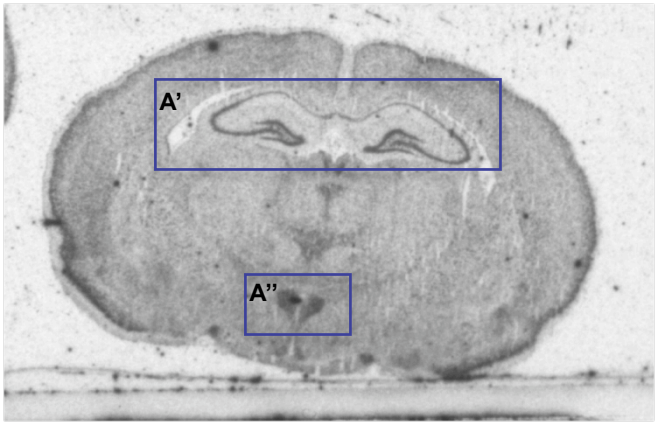
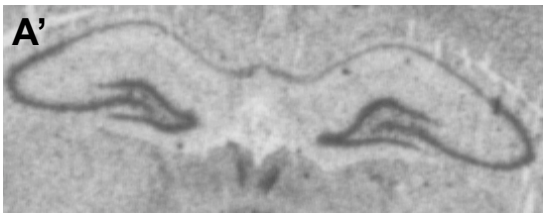
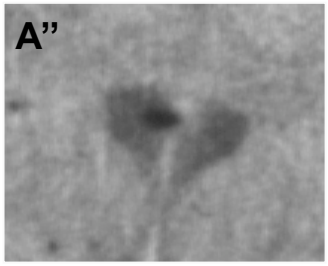


Figure 1

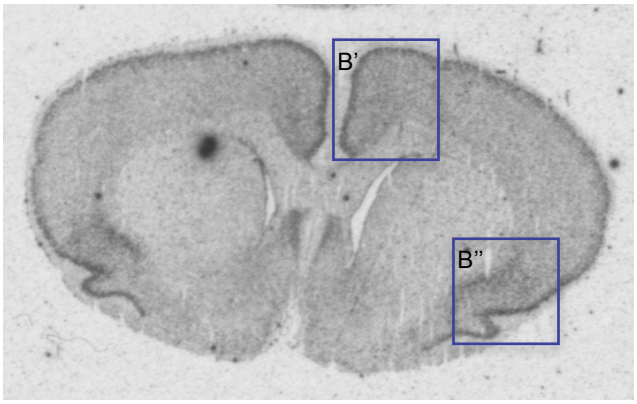
A

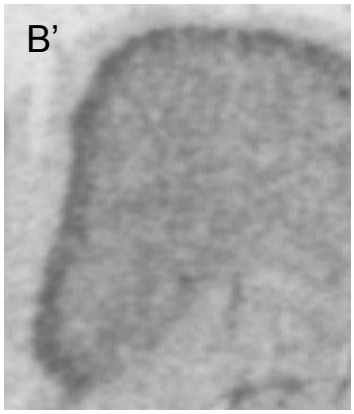






B





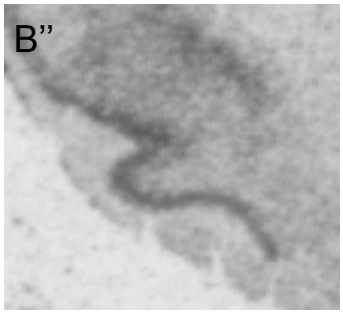
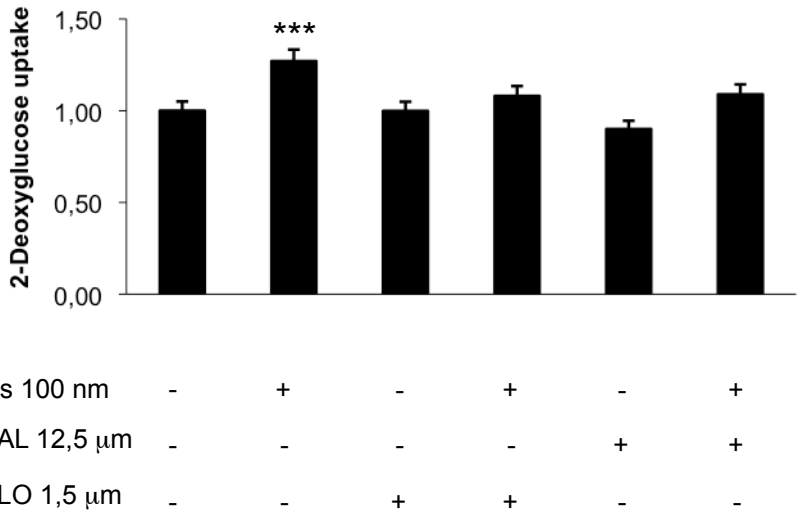


Figure 2

A

PC12



B

L6

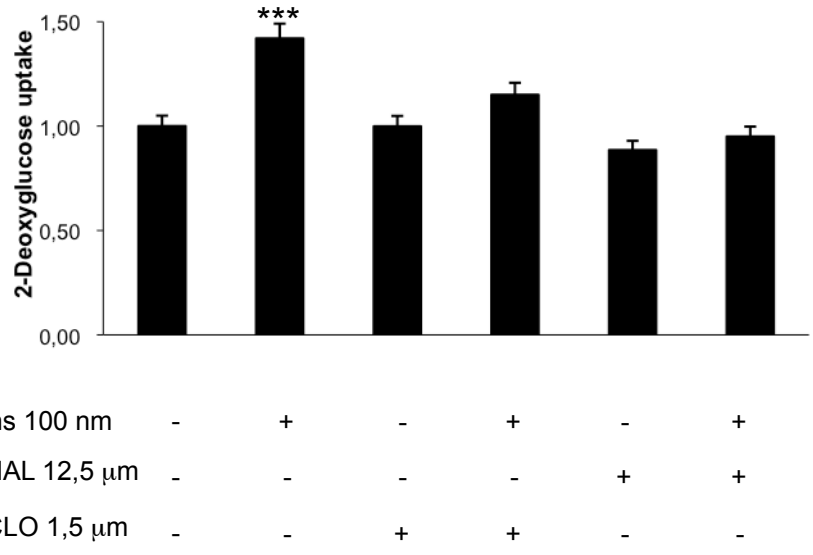
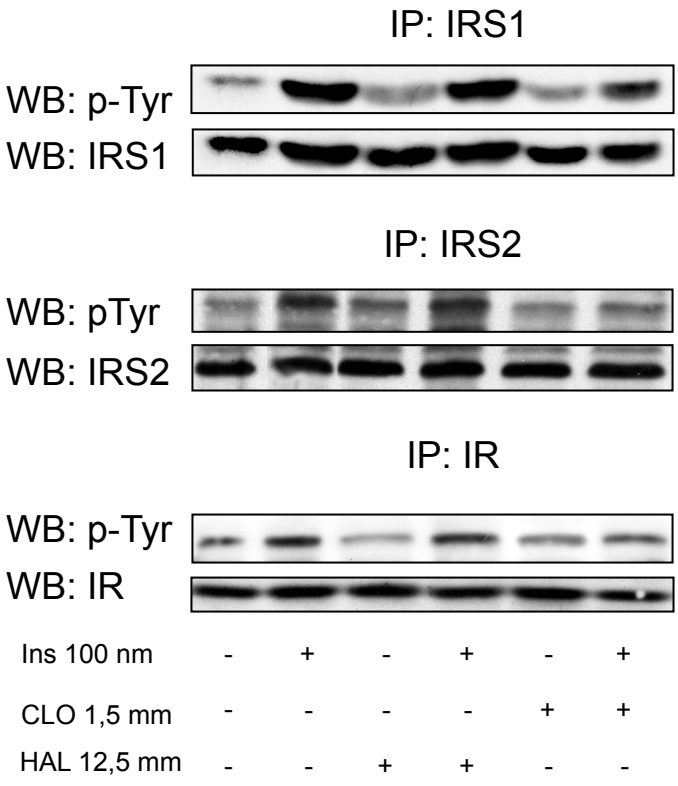


Figure 3

A


IP: IRS1
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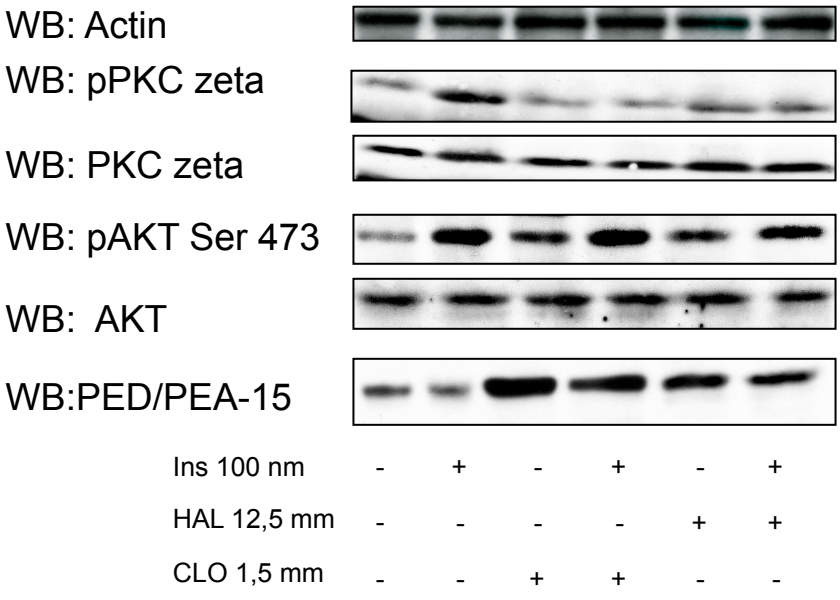
WB: Actin
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WB: PKC zeta
WB: pAKT Ser 473
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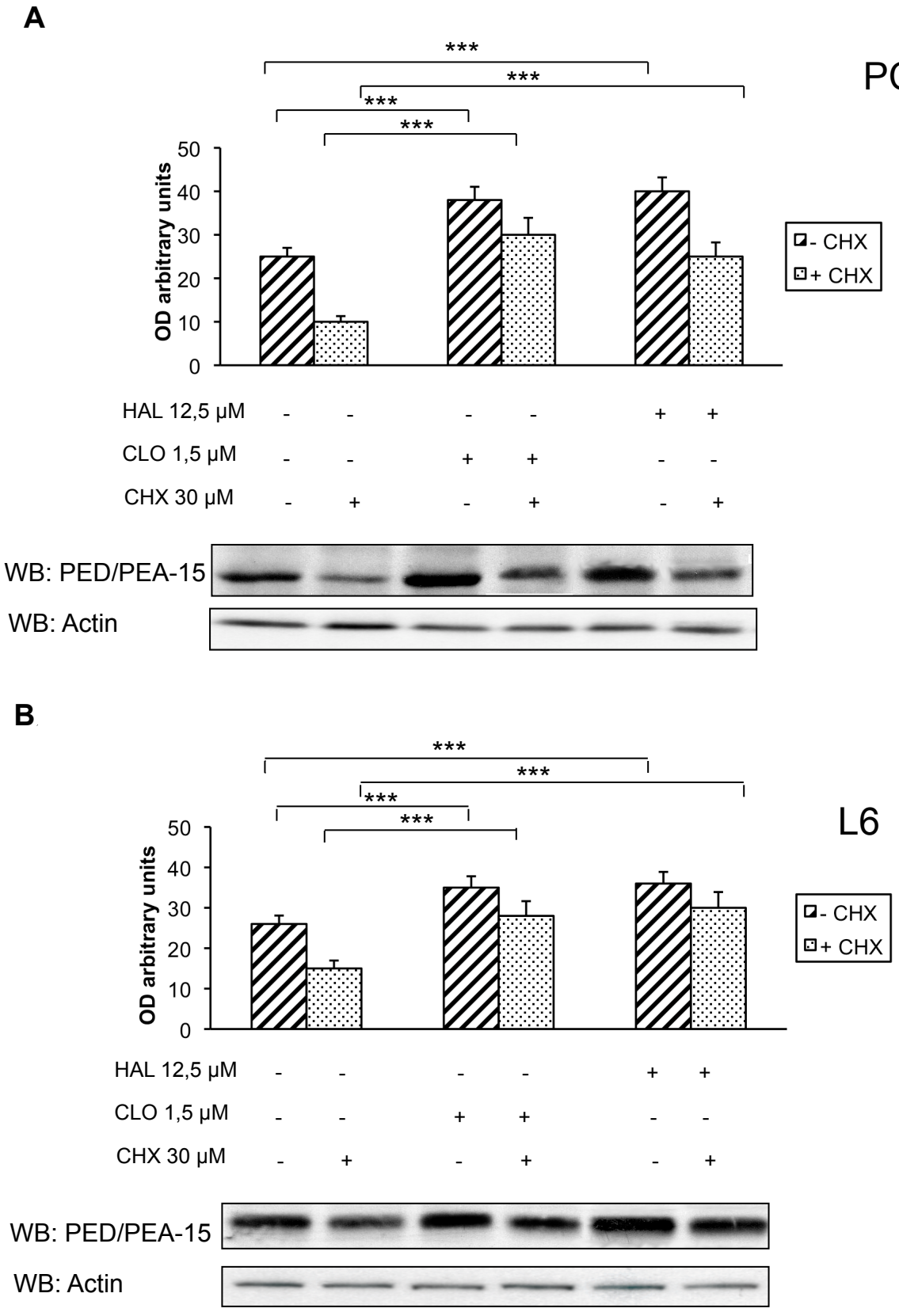
Figure 4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WB: PED/PEA-15
WB: Actin
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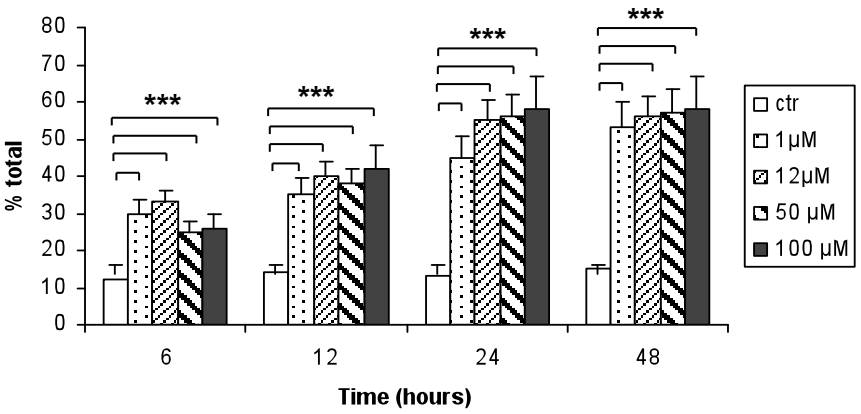
CLO 1,5 µM - - + + - -
CHX 30 µM - + - + - +
WB: PED/PEA-15
WB: Actin

Figure 5

PC12

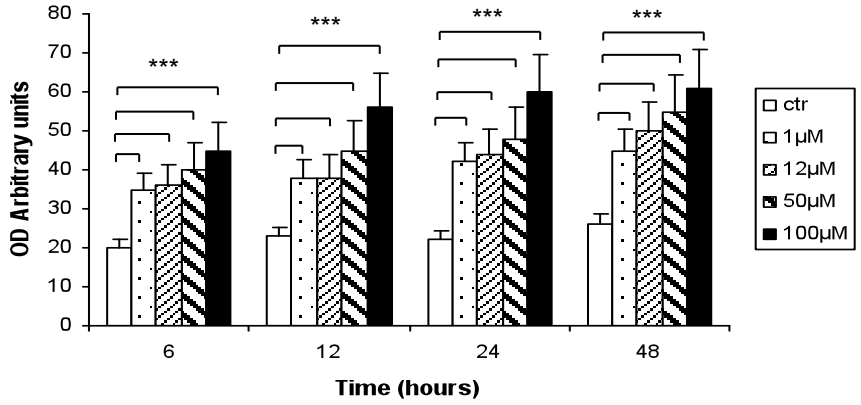
A

pAKT levels after HAL treatment



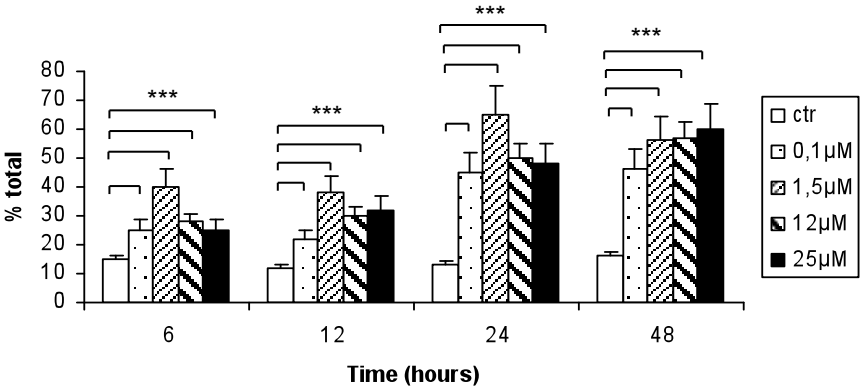
B

PED/PEA-15 levels after HAL treatment



C

pAKT levels after CLO treatment



D

PED/PEA-15 levels after CLO treatment

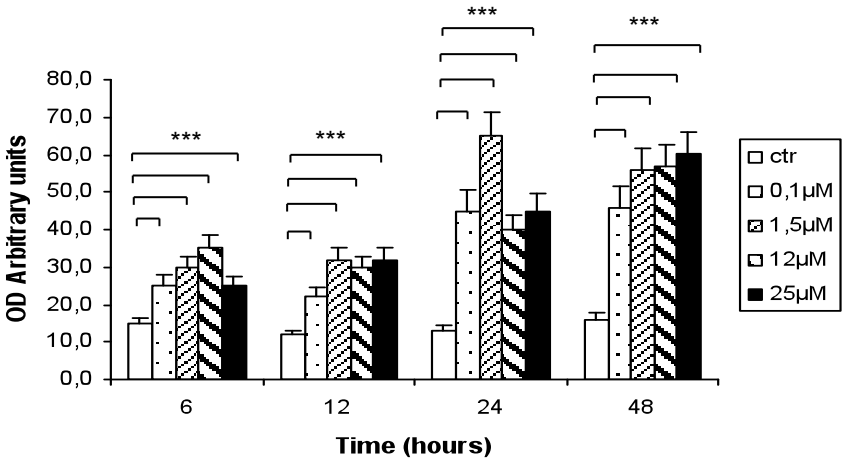
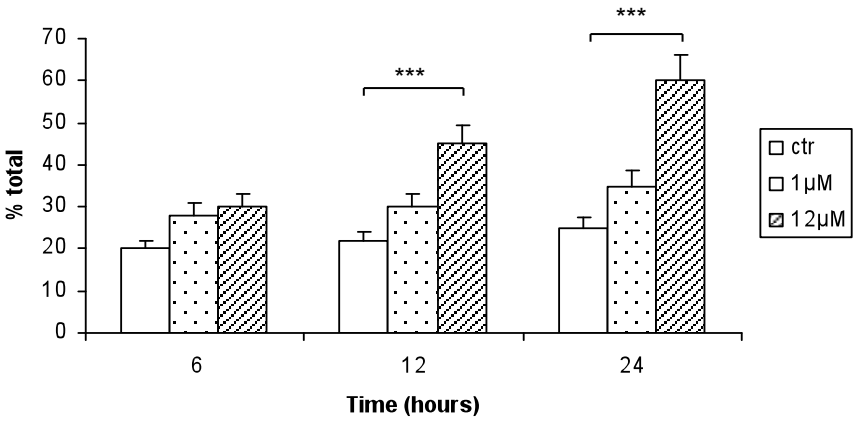


Figure 6

L6

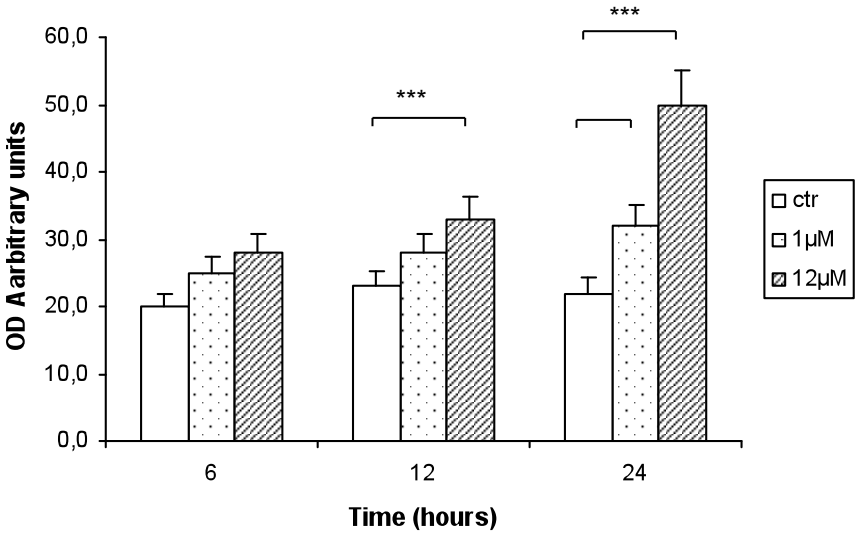
A

pAKT levels after HAL treatment



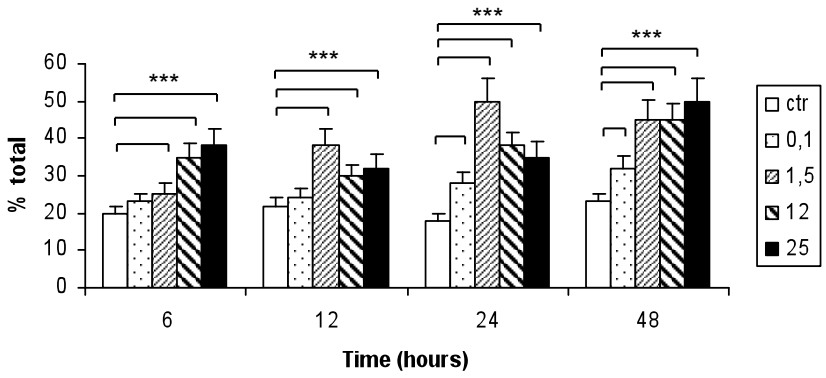
B

PED/PEA-15 levels after HAL treatment



C

pAKT levels after CLO treatment



D

PED/PEA-15 levels after CLO treatment

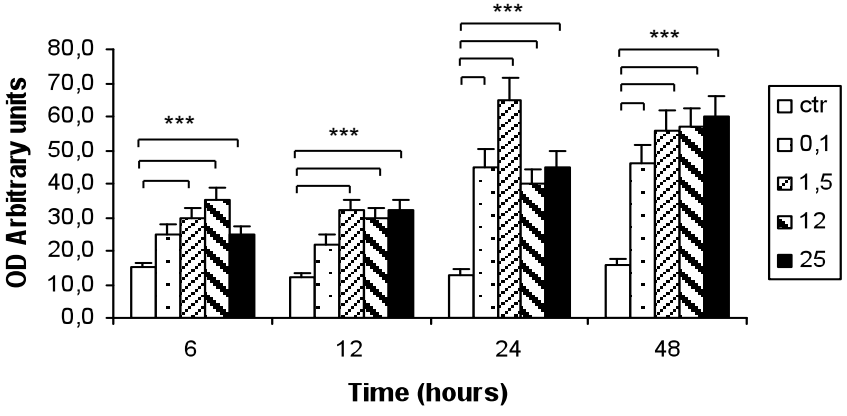
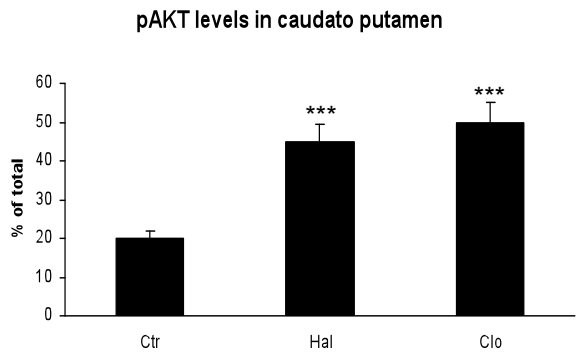
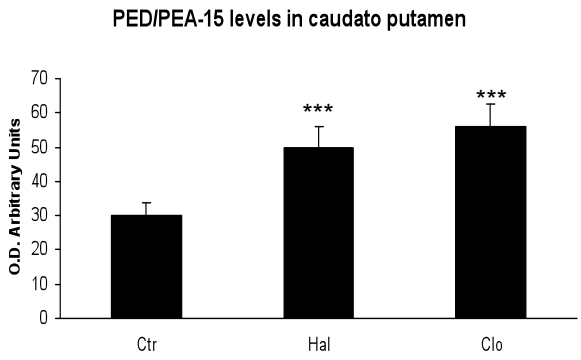


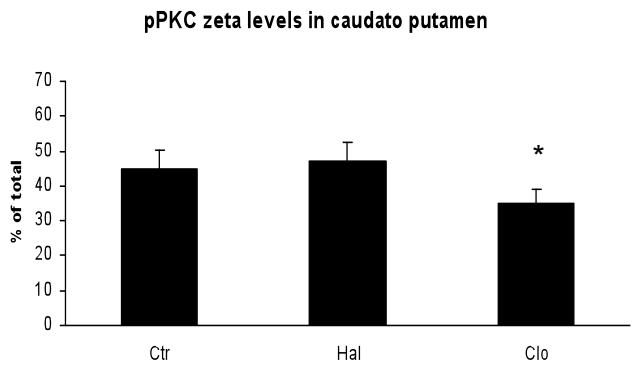
Figure 7

In vivo experiments

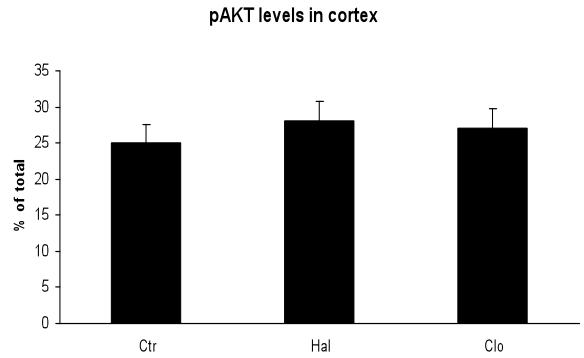
A

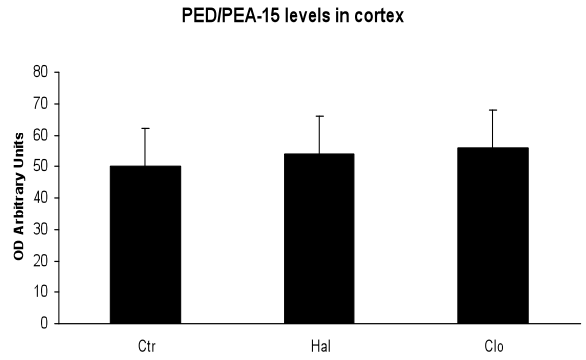






B





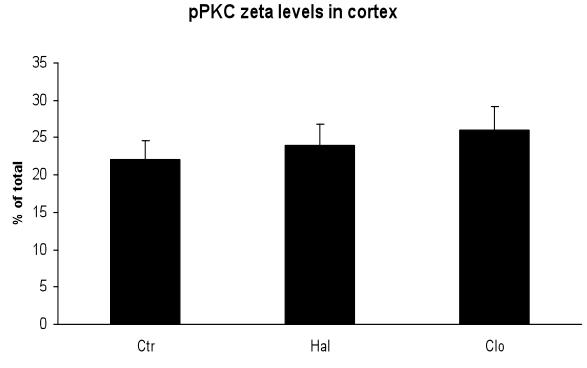
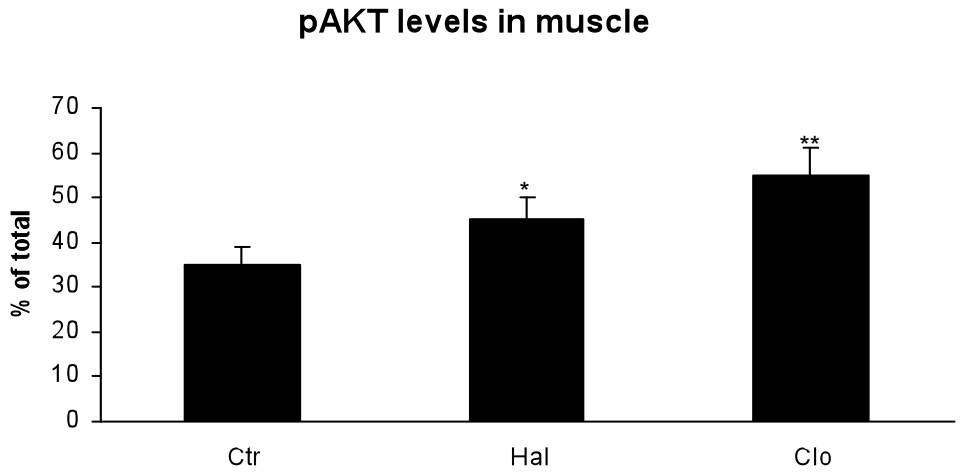
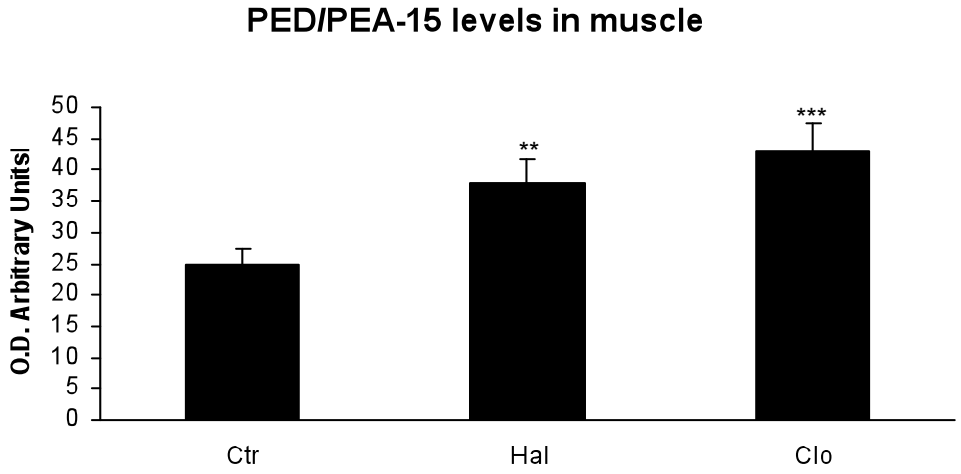


Figure 8


pAKT levels in muscle
% of total
70
60
50
40
30
20
10
0
*
**
Ctr
Hal
Clo


PED/PEA-15 levels in muscle
O.D. Arbitrary Units
50
45
40
35
30
25
20
15
10
5
0
**
***
Ctr
Hal
Clo

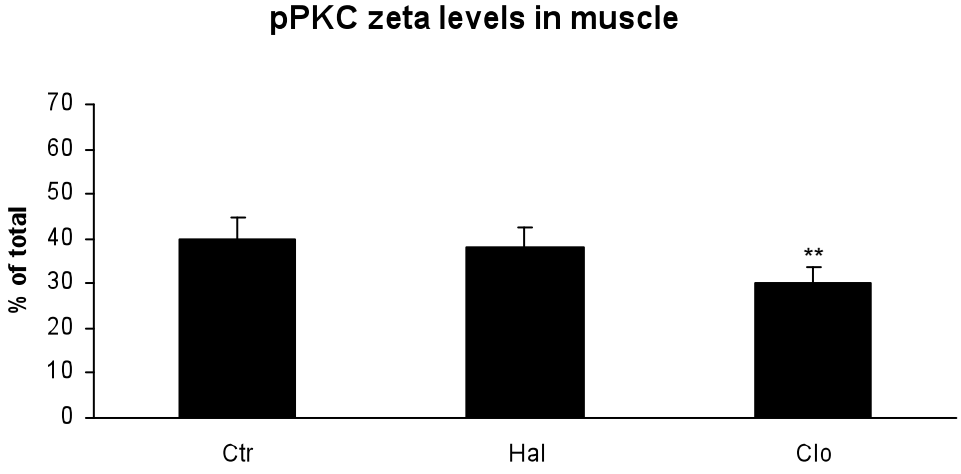

pPKC zeta levels in muscle
% of total
70
60
50
40
30
20
10
0
**
Ctr
Hal
Clo

Figure 9