B-esterase determination and organophosphate insecticide inhibitory effects in JEG-3 trophoblasts

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ABSTRACT

The placenta and trophoblasts express several B-esterases. This family includes acethylcholinesterase (AChE), carboxylesterase (CES) and butyrylcholinesterase (BChE), which are important targets of organophosphate insecticide (OP) toxicity. To better understand OP effects on trophoblasts, B-esterase basal activity and kinetic behavior were studied in JEG-3 choriocarcinoma cell cultures. Effects of the OP azinphos-methyl (Am) and chlorpyrifos (Cp) on cellular enzyme activity were also evaluated.

JEG-3 cells showed measurable activity levels of AChE and CES, while BChE was undetected. Recorded Km for AChE and CES were 0.33 and 0.26 mM respectively. Native gel electrophoresis and RT-PCR analysis demonstrated CES1 and CES2 isoform expression. Cells exposed for 4 and 24 h to the OP Am or Cp, showed a differential CES and AChE inhibition profiles. Am inhibited CES and AChE at 4 h treatment while Cp showed the highest inhibition profile at 24 h. Interestingly, both insecticides differentially affected CES1 and CES2 activities.

Results demonstrated that JEG-3 trophoblasts express AChE, CES1 and CES2. B-esterase enzymes were inhibited by in vitro OP exposure, indicating that JEG-3 cells metabolization capabilities include phase I enzymes, able to bioactivate OP. In addition, since CES enzymes are important for medicinal drug activation/deactivation, OP exposure may interfere with trophoblast CES metabolization, probably being relevant in a co-exposure scenario during pregnancy.

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1. Introduction

Organophosphates insecticides (OP) are worldwide used pesticides. Pesticides are designed to control pests, however once released into the environment they interact with both target and non-target species. In particular, the potential toxic effects of environmental OP exposure to the developing fetus represent a concern to the society and regulatory agencies (Shelton et al., 2014). Intrauterine environment is regarded as the first chemical exposure scenario in life. OP are lipophilic chemicals that can be absorbed by the placenta, and may cause alterations to the fetus (Akhtar et al., 2006; Shin et al., 2014), the placenta (Bulgaroni et al., 2013; Chiapella et al., 2013; Levario-Carrillo et al., 2004; Vera et al., 2012) and trophoblasts (Guiñazú et al., 2012; Ridano et al., 2012; Saulsbury et al., 2008). The placenta also serve as a *de facto* liver for the fetus, since it detoxifies a number of xenobiotics through metabolism enzymes such as phase I cytochrome P450 enzyme family, carboxylesterase family, and phase II N-acetyltransferase, and UDP-glucuronosyltransferase (Hakkola et al., 1998; Myllynen et al., 2007).

The cytochrome P450 enzyme family catalyzes OP bioactivation by oxidative desulfuration of the phosphorothionate to form the OP-oxon (Croom et al., 2010). The bioactivated OP-oxon acts as pseudo-substrate for a variety of serine hydrolases and covalently inhibits these enzymes by reaction with their active-site serine residue (Moser and Padilla, 2011). Thus, OP-oxon is a potent B-esterases inhibitor, as is the case for acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and carboxilesterases (CES). It has been recognized that OP-induced neurotoxicity depends mainly on AChE inhibition. However, there is a growing consensus that other serine hydrolases distinct from AChE may represent relevant targets for OP toxicity (Casida and Quistad, 2005; Pope, 1999). AChE is an enzyme located at neuromuscular and central cholinergic synapses, with the essential function of hydrolyzing ACh to limit the duration of cholinergic receptor activation (Colović et al., 2013). In turn, CES metabolize a broad range of endogenous as well as man-made chemicals and pharmaceuticals (Wheelock et al., 2008), since they hydrolyze carboxylic esters (Jokanović, 2001). Other cholinesterases such as BChE have a less clear (or multiple) physiological role (Karczmar, 2010).

Even though the placenta is a non-innervated organ, it expresses cholinergic components such as acetylcholine (ACh), choline acetyl-transferase (ChAT), AChE, muscarinic and nicotinic receptors (Bhuiyan et al., 2006). In non-nervous systems ACh signaling has been involved in the regulation of basic cell functions such as proliferation, differentiation, organization of the cytoskeleton, local release of mediators (*i.e.* nitric oxide, pro-inflammatory cytokines), locomotion, secretion and ciliary activity (Wessler et al., 2003). Recently Wessler et al. (2012), reported a specific anti-ChAT immunogold deposition in trophoblast cells within the cell membrane, microvilli, caveolae and the cytosol, suggesting that trophoblasts represent an ACh source in the placenta (Wessler et al., 2012).

Human choriocarcinoma cell lines such as JEG-3 cells have been extensively used for investigating xenobiotic *in vitro* effects in the placenta (Huang and Leung, 2009; Letcher et al., 1999; Ronco et al., 2010). Since cell line cultures are a good alternative for the *in vitro* study of xenobiotic toxic effects, the aim of this study was to measure and characterize AChE, CES and BChE in JEG-3 trophoblast cell line. The enzymological characterization consisted of activity measurements in the presence of specific substrates and inhibitors as well as the determination of enzyme kinetic parameters. The expression of CES isoenzymes by native gel electrophoresis followed by enzyme staining, and by conventional RT-PCR was also studied. In addition, the enzyme sensitivity to the OP azinphos-methyl and chlorpyrifos induced inhibition in the cell cultures, was analyzed.

2. Material and methods

2.1. Chemicals

Reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA), unless otherwise stated. The OP compounds azinphos-methyl – Am – (3-(dimethoxyphosphinothioylsulfanylmethyl)-1,2,3-benzotriazin-4-one) and chlorpyrifos –Cp- (O,O-diethyl O-3,5,6 trichloro-2-pyridyl phosphorothioate) were of standard analytical grade according to the supplier (Chem Service, West Chester, PA, USA) with a 99.5% purity.

2.2. Cell culture

JEG-3 choriocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). JEG-3 cells were grown in Dulbecco modified Eagle medium – DMEM – (GIBCO, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine and 1 mM sodium pyruvate, at 37 °C in 5% CO_2. Cells were plated at 2×10^6 cells per 25 cm^3 flask and incubated under standard cell culture conditions until achieving an 80–90% confluence. Cells were trypsinyzed with 0.1% trypsin/EDTA in PBS pH 7.4, washed twice (PBS pH 7.4) and plated. In order to perform the exposure treatments, the cell was cultured in culture medium containing the insecticides for 4 and 24 h.

2.3. Insecticide treatment

JEG-3 cells were grown in 10 cm Petri dish at an initial concentration of 1×10^6 cells/10 ml. After achieving 80% confluence, the culture medium was removed, and replaced with 10 ml culture media containing the insecticide. Then, the cultures were incubated for 4 or 24 h prior to harvest and analysis. The insecticide concentrations assayed were 0.1, 1, 10 and 100 μM. These chemicals were dissolved in DMSO and freshly prepared prior to use. Control cells contained equivalent quantities of DMSO (0.02%). The concentration range selected for the experimental design agrees with previously published *in vitro* models (Chiapella et al., 2013; Guiñazú et al., 2012; Saulsbury et al., 2008), and the lower concentrations used were in the range of the OP levels determined in human samples (Ostrea et al., 2009; Pluth et al., 1996; Sanghi et al., 2003).

2.4. Acetylcholinesterase, butyrylcholinesterase and carboxylesterase activities

The enzyme activities were measured in JEG-3 cell lysates. Briefly, cell lysates (3×10^6 cells) were obtained in 500 μl of 0.1 M phosphate buffer pH 6.5 containing 0.5% Triton X-100. AChE (EC 3.1.1.7) activity was determined following the Voss and Sachsse method (Voss and Sachsse, 1970). Measurements were carried out at 412 nm using acetylthiocholine as substrate, with a molar absorption coefficient of 13.6 mM^{-1} cm^{-1}. AChE activity was expressed as nmol of hydrolyzed substrate per minute and mg protein. BChE (EC 3.1.1.8) activity was determined following the Voss and Sachsse method (1970). Measurements were carried out at 412 nm using butyrylthiocholine as substrate, with a molar absorption coefficient of 13.6 mM^{-1} cm^{-1}. CES (EC 3.1.1.1) activity was determined by the hydrolysis rate of 2-naphthyl acetate at 550 nm, in a 96-well microtiter plate by a colorimetric assay previously described (Vera et al., 2012). CES activity was expressed as nmol of hydrolyzed substrate per minute and mg protein. Under the initial conditions for AChE and CES assays, preferences for the substrates acetylthiocholine and 2-naphthyl acetate, were examined by incubation of the crude homogenate with varying concentrations of the substrates (data not shown). CES activity was performed in the presence of BW284c51, in all assays. Total protein content was quantified by the Bradford method. All measurements were performed in triplicate and a mean value was considered for the calculations. AChE and BChE activity measurements were carried out in a UV/Vis 1603 Shimadzu Spectrophotometer. The kinetic parameters Vmax, Km and Ki for AChE were calculated with the equation $Y = Vmax * X/(Km + X *(1 + X/Ki))$, whereas Vmax and Km for CES were calculated with the Michaelis–Menten equation $Y = Vmax*X/(Km + X)$, using the Graphpad Prism software.

2.5. Acetylcholinesterase and carboxylesterase inhibition

AChE and CES activities were characterized using the cell lysate as esterase source. Selective substrates and inhibitors were used to distinguish between AChE and CES activities. The sensitivity of AChE activity to selective inhibitors was investigated by incubation of the sample at varying concentrations of eserine, a selective cholinesterase inhibitor; or BW284c51, a selective inhibitor of mammalian AChE activity. Inhibitors were initially prepared as 5 mM stock solutions in 0.1 M Na-phosphate buffer (pH 7.0) containing 50% ethanol. Enzyme inhibition percentages were calculated from control samples, free of inhibitors, which were incubated under the same experimental conditions than inhibitor treated samples.

2.6. Native electrophoresis of carboxylesterase isozymes

Native polyacrylamide gel electrophoresis (PAGE) was carried out on a Bio-Rad Tetra Cell Electrophoresis Unit (Bio-Rad, USA). Cell lysate (150 μg protein) was loaded on 4% stacking and 10% separating 1.5 mm polyacrylamide gel. The electrophoresis was conducted at 15 V for 30 min and subsequently at 150 V until the tracking dye, bromophenol blue, reached the bottom of the gel. The running buffer was 25 mM Tris, plus 192 mM glycine (pH 8.6). Bands, corresponding to CES activity, were visualized by incubation with a staining (100 mM Na-phosphate buffer, pH 6.4) solution containing, 500 μl 2-naphthyl acetate (2%) and 0.025 g of fast garnet salt, which was prepared immediately before use. After staining for 30 min at room temperature, the gels were washed with an aqueous solution containing 30% methanol and 10% acetic acid. Stained gels were scanned and band relative intensity was determined by densitometry analysis (Gel Pro Analyzer 3.2 program).

2.7. Carboxylesterase isoforms mRNA expression

Total RNA was isolated from cultured JEG-3 cells using Tri reagent (Genbiotech, Buenos Aires, Argentina) according to the manufacturer's instructions. To obtain cDNA, mRNA was reverse-transcribed using Thermo Scientific RevertAid Reverse Transcriptase (Fermentas) with oligo-dT primers (Thermo Scientific &D Systems Europe Ltd., Abingdon, UK) for 60 min at 42 °C, in a volume of 20 μl. PCR reactions were performed to detect expression of transcripts encoding CES1, CES2, CES3 and cyclophilin A. PCR reactions were carried out in 20 μl buffered medium containing 3 μl cDNA, 0.5 μl CES primers (0.25 mM), 2 μl buffer taq (Thermo Scientific); 0.2 μl dNTPs, 1 μl MgCl$_2$ (25 mM) and 0.15 μl taq DNA polymerase (Thermo Scientific). Reactions included an initial denaturing step of 3 min at 95 °C, followed by 35 cycles with 30 s at 95 °C, 30 s at 56 °C, and 72 °C for 30 s on a BIOER XP cycler instrument. Lastly, the reaction was completed at 72 °C for a further 10 min.

The specific primers for each gene are as follows: CES1 forward 5-AGAGGAGCTCTTGGAGACGACAT-3 and reverse 5-ACTCCTGCTTGTTA ATTCC GACC-3; CES2 forward 5-AACCTGTCTGCCTGTGACCAAGT-3 and reverse 5-ACATCAGCAGCGTTAACATTTTCTG-3; CES3 forward 5-CTGG TCCTTAGCAAG AAGCTGAAA-3 and reverse 5-CATTTGGCTTGTGCGTCC GAGTT-3. Internal control, Cyclophilin A forward 5-GTCAACCCCACCGT CTTCTT-3 and reverse 5-CTGCTGTCTTTGGGACCTTGT-3 (Bulgaroni et al., 2013).

2.8. Statistical analysis

Comparison between groups was performed using one-way ANOVA followed by the *post hoc* Tukey's multiple comparison test. A value of $p < 0.05$ was considered to be significant. The enzyme kinetic parameters, IC$_{50}$ values and inhibition parameters were calculated using the Graphpad Prism software.

3. Results

3.1. Acetylcholinesterase, carboxylesterase and butyrylcholinesterase activities

The basal esterase activity in JEG-3 cells was determined by the hydrolysis of acetylthiocholine for AChE, 2-naphthyl acetate for CES and butyrylthiocholine for BChE. Fig. 1A, shows that AChE and CES activities can be accurately measured, however BChE activity was not detected under these experimental conditions.

In order to examine the esterase characteristics, AChE and CES kinetic parameters were determined. Km and Vmax values are listed in Table 1.

Fig. 1B and C shows AChE and CES kinetic profile, respectively. AChE activity was inhibited by substrate (Fig. 1B), while non-substrate inhibition was observed for CES activity at substrate concentration as higher as 7.68 mM (Fig. 1C).

3.2. Carboxylesterase and acetylcholinesterase sensitivity to inhibitors

AChE activity decreased in a dose dependent manner with eserine, a cholinesterase specific inhibitor, or BW284c51, an AChE specific inhibitor, and reached 50% inhibition with 300.2 nM eserine and 31.6 nM BW284c51 (Fig. 2A).

No significant effects were observed on CES activity in the presence of eserine or BW284c51 (Fig. 2B) under these experimental conditions.

3.3. Carboxylesterase isoform expression

Since CES total activity may be the result of different enzyme isoforms (Imai, 2006), native PAGE gel was performed. Fig. 3A, shows native PAGE gel stained by esterase activity hydrolyzing 2-naphthyl acetate. The gel analysis showed the activity of several CES isoforms in
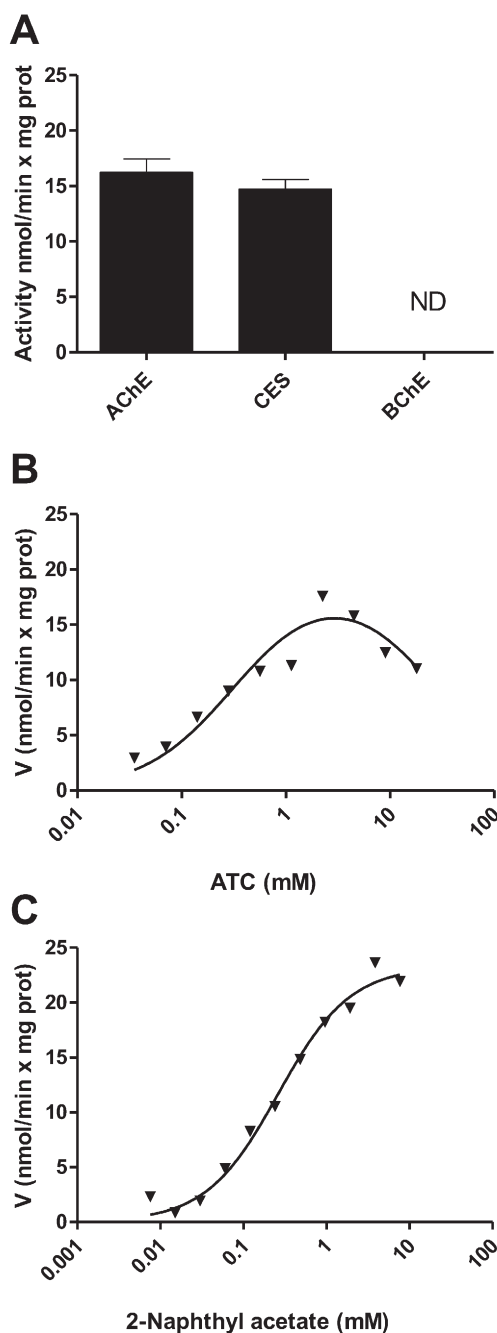

Fig. 1. B-esterase activity in JEG-3 cells. (A) Activity levels of acetylcholinesterase (AChE), carboxylesterase (CES) and butyrylcholinesterase (BChE) in JEG-3 cells. The data are presented as mean ± SEM, from at least three different assays. ND, not detected. (B) AChE kinetic parameters were determined using acetylthiocholine (ATC) as substrate. (C) CES kinetic parameters were determined using 2-naphthyl acetate as substrate. Each symbol is the mean of three determinations with each substrate.

Table 1
Acetylcholinesterase and carboxylesterase kinetic parameters in JEG-3 cells.

	Vmax	Km	Ki
Acetylcholinesterase	19.07 ± 1.33	0.33 ± 0.06	26.2 ± 7.8
Carboxylesterase	23.31 ± 0.61	0.26 ± 0.03	N.I.

Vmax are expressed as nmol/min/mg protein (mean ± SE). Also, for acetylcholinesterase and carboxylesterase. Km and Ki (inhibition constant) are expressed as mM (mean ± SE). Values were obtained by non-linear regression of kinetic plots. N.I.: non-substrate inhibition observed.
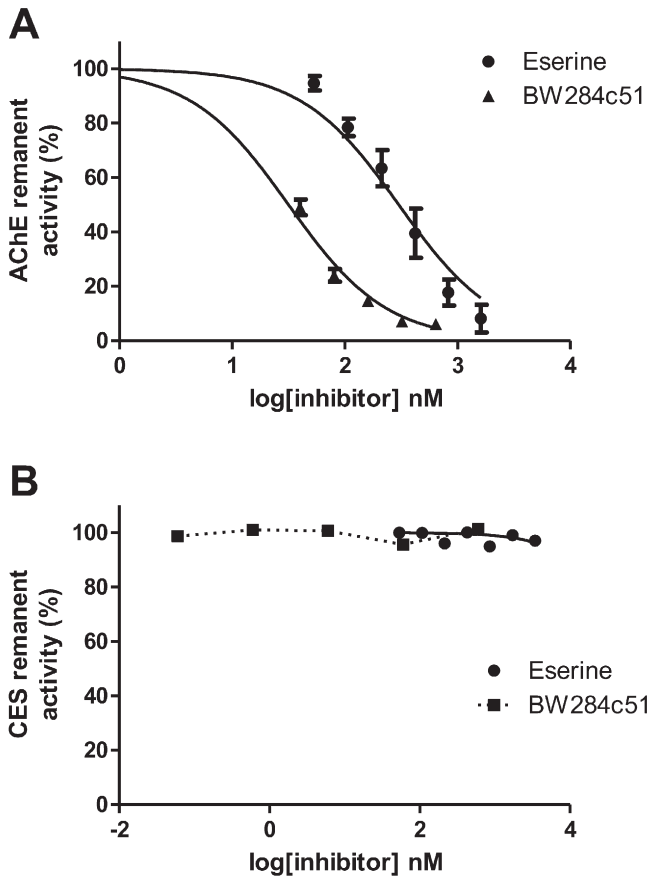
Fig. 2. Effects of eserine and BW284c51 on acetylcholinesterase and carboxylesterase activities. Cell lysates were incubated with different concentrations of eserine and BW284c51 for 30 min. (A) Acetylcholinesterase (AChE) inhibition percentages. (B) Carboxylesterase (CES) inhibition percentages. The graphs show the enzyme inhibition percentages with respect to the enzyme activity of untreated cells. Each symbol represents two determinations.

JEG-3 cells. Samples were also treated with eserine or BW284c51 no band changes were observed (data not shown).

In order to determine which isoform is expressed, RT-PCR analysis with specific primers for CES1, CES2 and CES3, human isoforms, was
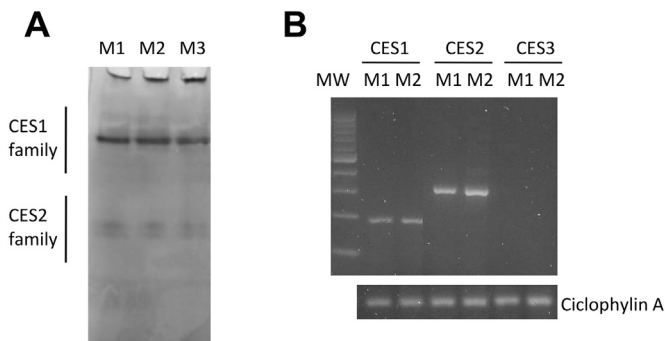

Fig. 3. Carboxylesterase isoforms in JEG-3 cells. (A) Non denaturing activity gel electrophoresis of JEG-3 cells. Three different samples of JEG-3 cells maintained in basal conditions were processed (M1, M2 and M3). 150 µg of cell lysates were separated on 10% non-denaturing gel and stained for CES activity with 2-naphthyl acetate as substrate and fast garnet as colorant. (B) JEG-3 cells mRNA was processed by conventional RT-PCR and CES1, CES2 and CES3 were amplified by PCR specific primers. PCR products were separated by 2% agarose gel electrophoresis. JEG-3 cells in the basal condition were processed (M1 and M2). Samples shown are lane MW (100-bp DNA markers), lane 1 M1 CES1, lane 2 M2 CES1, lane 3 M1 CES2, lane 4 M2 CES2, lane 5 M1 CES3, lane 6 M2 CES3. Ciclophylin A was processed as an internal standard in all samples.

performed. In accordance with native PAGE-gel results, RT-PCR showed CES1 and CES2 mRNA expression by JEG-3 cells (Fig. 3B).

3.4. Carboxylesterase and acetylcholinesterase sensitivity to organophosphate insecticide inhibition

To investigate AChE and CES sensitivity to OP inhibition, JEG-3 cell cultures were exposed to 0.1, 1, 10 and 100 µM Am or Cp for 4 and 24 h, and enzyme activities were recorded. Enzymes IC50 are listed in Table 2.

Am treatment for 4 h significantly inhibited AChE at all the concentrations assayed. In contrast, a 24 h Am treatment significantly decreased AChE at 10 and 100 µM (Fig. 4A). CES activity was significantly inhibited after 4 h incubation with Am 1, 10 and 100 µM. After 24 h treatment, only Am 10 and 100 µM significantly inhibited CES activity (Fig. 4B). To determine whether CES isoenzyme exhibited a different sensitivity to Am inhibition, native PAGE gel was performed. CES2 showed the highest activity inhibition after 24 h treatment (Fig. 4C).

Cp treatment for 4 h induced a significant AChE inhibition at the highest concentration assayed. In contrast, a 24 h Cp treatment significantly decreased AChE activity at all concentration studied (Fig. 5A). CES activity wasn't significantly affected after 4 h Cp incubation, while after 24 h treatment all concentration assayed significantly inhibited CES activity (Fig. 5B). To determine whether CES isoenzyme exhibited a different sensitivity to Cp inhibition, native PAGE gel was performed; and CES1 showed a highest activity inhibition after 24 h treatment (Fig. 5C).

4. Discussion

This work reports that JEG-3 trophoblasts showed B-esterase activity, by expressing AChE and CES enzymes. Since the classic OP toxicity pathway is AChE inhibition (Colović et al., 2013), AChE basal kinetic profile as well as sensitivity to OP inhibition, was tested. AChE activity followed a kinetic response inhibited by high substrate concentration. Also, AChE activity was highly sensitive to the classic inhibitors eserine and BW284c51. These results are consistent with data reported in placenta *in vitro* models (Sánchez et al., 2015), and the cholinesterase activity levels found are in accordance with those reported in different cell lines (Das and Barone, 1999; Marinovich et al., 1996). The placenta is a tissue without innervation yet it contains all cholinergic system components. This system may regulate developmental processes relevant to placental growth, blood flow and fluid volume in the vessels and vascularization, with ACh acting as a signaling molecule (Bhuiyan et al., 2006). It has been demonstrated that cytotrophoblast is a source for ACh in the placenta, and that ChAT is expressed by Bewo and JAR trophoblast cell lines (Sastry and Janson, 1997). AChE activity has been found in the syncytiotrophoblast, cytotrophoblast cells, endothelial cells and the media of fetal blood vessels of the human placenta (Hahn et al., 1993). Recently it has been postulated that cellular AChE expression contributes to a generalized mechanism for polarized membrane protrusion and migration in all adherent cells (Anderson et al., 2008). Then, AChE inhibition or modulation may have further implications in placental development.

This work reports for the first time that JEG-3 trophoblasts possessed CES enzyme activity. Moreover, CES activity resulted from the

Table 2
Acetylcholinesterase and carboxylesterase IC50 after insecticide treatments in JEG-3 cells.

	Acetylcholinesterase		Carboxylesterase	
	4 h	24 h	4 h	24 h
Chlorpyrifos	73.6 ± 1.53	0.15 ± 1.65	N.D.	4.5 ± 1.74
Azinphos-methyl	1.7 ± 1.49	16.3 ± 1.72	52.2 ± 1.52	14.3 ± 2.05

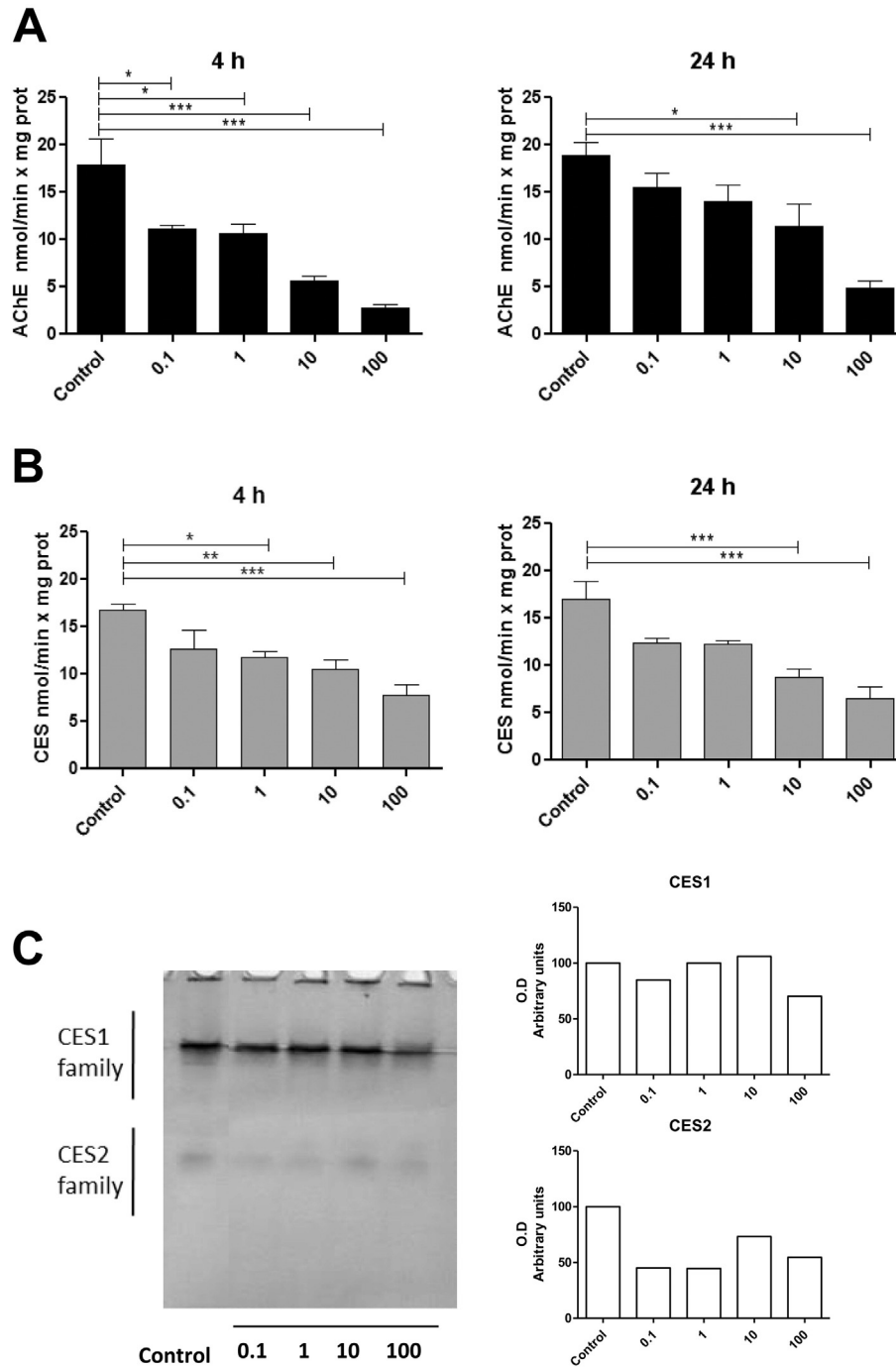IC50 are expressed as µM (mean ± SE). N.D., not determined.

Fig. 4. Acetylcholinesterase and carboxylesterase activity in JEG-3 cells after azinphos-methyl organophosphate insecticide exposure. Cells were exposed to azinphos-methyl (Am) at 0.1, 1, 10 and 100 μM, for 4 or 24 h. (A) Acetylcholinesterase (AChE) activity was determined with acetylthiocholine (ATC) as substrate. AChE activity is expressed as nmol hydrolyzed substrate/min per mg protein. (B) Carboxylesterase (CES) activity was determined with 2-naphthylacetate as substrate. CES activity is expressed as nmol hydrolyzed substrate/min per mg protein. The graph shows the mean activity ± SEM, from three different experiments, with each sample processed as triplicates. ***p < 0.0001, **p < 0.001, *p < 0.05 one way ANOVA followed by Tukey's multiple comparison test. (C) Non denaturing activity gel electrophoresis of JEG-3 cells was exposed to Am for 24 h. 150 μg of cell lysates were separated on 10% non-denaturing gel and stained for CES activity with 2-naphthyl acetate as substrate and fast garnet as colorant. Samples shown are lane 1 control cells (DMSO 0,02%), lane 2 cells exposed to 0.1 μM, lane 3 cells exposed to 1 μM, lane 3 cells exposed to 10 μM, and lane 4 cells exposed to 100 μM Am. (D) CES1 and CES2 band relative intensitieswere estimated by densitometry analysis (Gel Pro Analyzer 3.2 program).

co-expression of the different isoforms CES1 and CES2. Five families of mammalian CES have been described, including CES1, the major liver enzyme, CES2 mainly expressed in the small intestine, CES3 expressed in brain, liver and colon, CES5, a major urinary protein of the domestic cat also present in human tissues and CES6 a predicted carboxylesterase-like enzyme in the brain (Holmes et al., 2010; Satoh and Hosokawa, 2006). The placenta expresses CES1, CES2 and CES3 isoforms (Yan et al., 1999). Among human CES, the two major isoforms CES1 and CES2 are

the most studied and relevant (Redinbo and Potter, 2005). Native PAGE anaysis showed the presence of different bands in JEG-3 cells, taking into consideration the CES native gel reported by others (Ross and Crow, 2007; Sanghani et al., 2003; Taketani et al., 2007), CES1 and CES2 families were active in these cells. Also, the RT-PCR results confirmed CES1 and CES2 expression, while CES3 mRNA was not detected.

Enzyme inhibition after Am or Cp incubation followed a time and dose response dependence particular for each OP. Am was an efficient
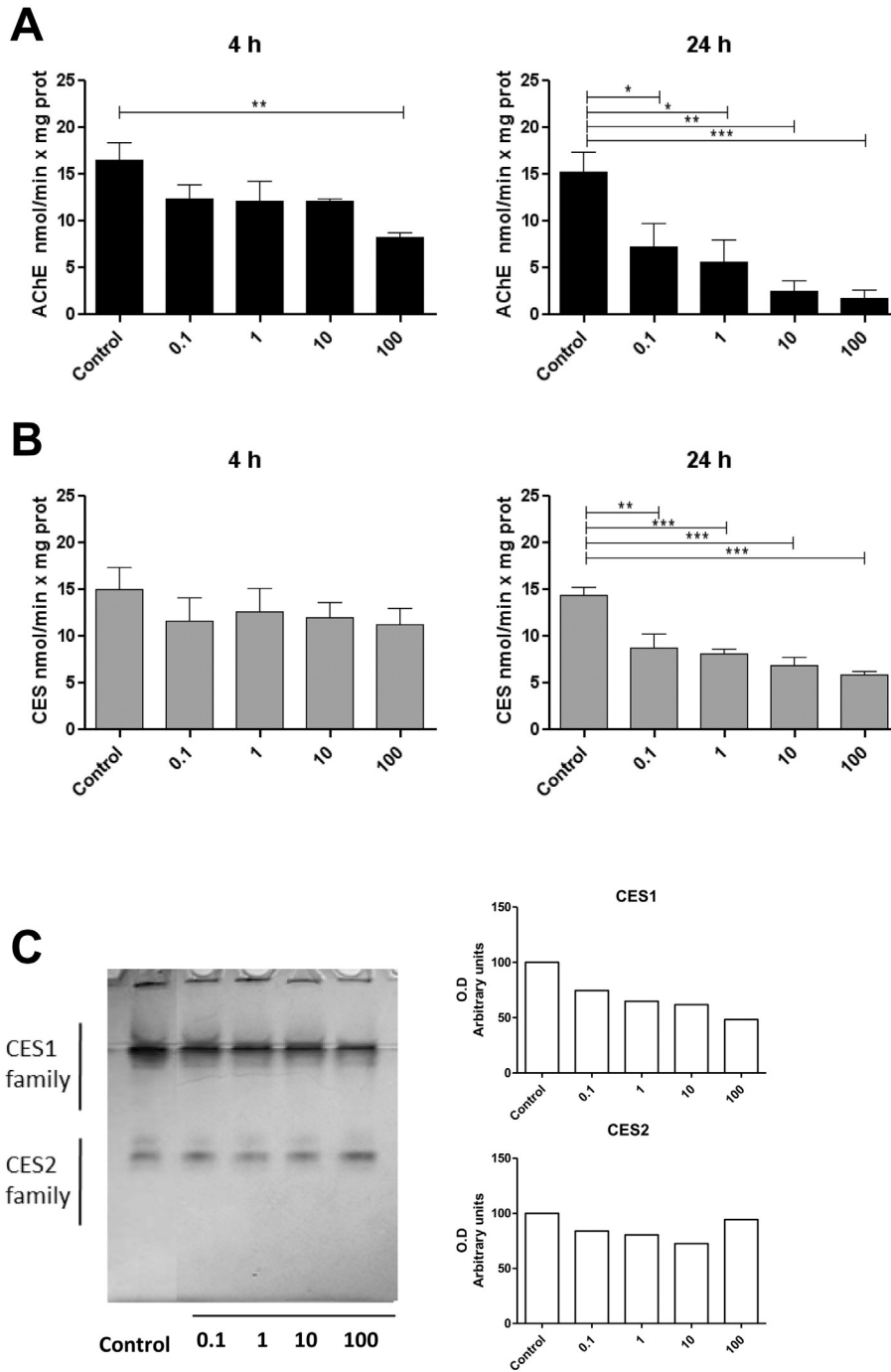
Fig. 5. Acetylcholinesterase and carboxylesterase activity in JEG-3 cells after chlorpyrifos organophosphate insecticide exposure. Cells were exposed to chlorpyrifos (Cp) at 0.1, 1, 10 and 100 μM, for 4 or 24 h. (A) Acetylcholinesterase AChE activity was determined with acetyltiocholine (ATC) as substrate. AChE activity was expressed as nmol hydrolyzed substrate/min per mg protein. (B) Carboxylesterase CES activity was determined with 2-naphthyl acetate as substrate. CES activity was expressed as nmol hydrolyzed substrate/min per mg protein. The graph shows the mean activity ± SEM, from three different experiments, with each sample processed as triplicates. ***$p < 0.0001$, **$p < 0.001$, *$p < 0.05$ one way ANOVA followed by Tukey's multiple comparison test. (C) Non denaturing activity gel electrophoresis of JEG-3 cells was exposed to Cp for 24 h. 150 μg of cell lysates were separated on 10% non-denaturing gel and stained for CES activity with α-naphthyl acetate as substrate and fast garnet as colorant. Samples shown are lane 1 control cells (DMSO 0,02%), lane 2 cells exposed to 0.1 μM, lane 3 cells exposed to 1 μM, lane 3 cells exposed to 10 μM, lane 4 cells exposed to 100 μM Cp. (D) CES1 and CES2 band relative intensities were estimated by densitometry analysis (Gel Pro Analyzer 3.2 program).

AChE inhibitor at 4 h, while Cp developed the most efficient inhibition profile at 24 h. CES activity showed a similar amount of inhibition compared to AChE inhibition at 24 h, however at a shorter insecticide exposure period CES was less inhibited than AChE. It has been postulated that A- and B-esterases such as paraoxonase and CES, respectively have OP detoxification activities and therefore may exert protective functions with respect to AChE inhibition, nevertheless their detoxification patterns are chemical-specific (Moser and Padilla, 2011). However

in this experimental model remains unclear whether CES may exert a protective function and additional studies are needed.

Interestingly, the results shown also indicated that both insecticides developed a distinct time response for B-esterase inhibition. Since the OP used in these assays were not the bioactivated metabolites, B-esterase inhibition may be explained by the metabolic system expressed by these cells. These results indicated that JEG-3 trophoblasts have the metabolic machinery necessary for OP bioactivation, as

suggested previously (Chiapella et al., 2013; Guiñazú et al., 2012). Cytochrome P450 enzymes (CYP450) were not investigated in this work, however data reported by others demonstrate the expression of some CYP450 isoforms by JEG-3 cells, such as CYP19, CYP1A1, CYP1A2 and CYP3A4 (Li et al., 1998; Pavek et al., 2007). Probably these and other CYP isoforms would be capable of OP bioactivation/deactivation in JEG-3 cells, which may partly account for the differences observed between Am and Cp. Results shown may indicate that Am is more rapidly bioactivated than Cp, thus enzymes are at first inhibited by Am. It is also possible that Am is detoxified earlier than Cp, so AChE inhibition decreases over time. AChE spontaneous reactivation was reported to occur faster with dimethyl phosphates such as Am, compared with diethyl phosphates such as Cp (Jokanović, 2009), thus spontaneous reactivation may partly contribute for the differences observed. A more complicated scenario with an interlinked balance between OP bioactivation/deactivation, along with enzyme activity/expression induction (metabolism and B-esterase enzymes) may also be plausible.

CES activity after OP exposure was also analyzed in native PAGE gel, a non-clear correlation with biochemical inhibition was observed, indicating that enzyme regulation mechanism other than inhibition may also be triggered by OP incubation. Nevertheless, the inhibition profile observed for CES1 and CES2 isoenzymes clearly depended on the OP assayed. In this sense, CES2 was sensitive to Am induced inhibition, while CES1 was efficiently inhibited by Cp treatment. CES are members of the a/b hydrolase fold family and play an important role in the biotransformation of a variety of ester-containing drugs, pesticides, and prodrugs such as angiotensin-converting enzyme inhibitors (temocapril, cilazapril and imidapril), anti-tumor drugs (irinotecan (CPT-11) and capecitabine), and narcotics (cocaine, heroin and meperidine) (Imai, 2006). Since placental CES have been shown to be a target of OP toxicity (Bulgaroni et al., 2013; Vera et al., 2012), changes in CES activity/expression induced by environmental xenobiotic exposure may be relevant, not only for the toxic OP consequences but also for drug–drug interactions. It has been postulated that CES exert protective functions, in the case of OP intoxication they are irreversibly inactivated by the OP-oxon and less pesticide molecules remain available for AChE inhibition. Also CES are also responsible for the inactivation of therapeutic drugs (Redinbo and Potter, 2005), their inactivation may have further adverse consequences. Recently it has been demonstrated that CES1 is the major isoform involved in the hydrolysis of phospho-NSAIDs whereas CES2 is active toward phospho-aspirin, phospho-tyrosol-indomethacin, and phospho-naproxen (Wong et al., 2012). Human CES1A and CES2 govern the pharmacokinetic behaviors of most prodrugs, and the inhibitory effect on their activities by chemicals is vital for drug potency (Zhang et al., 2014a).

5. Conclusion

Pregnant women are treated with a significant number of prescription and non-prescription medications (Rubinchik-Stern and Eyal, 2012), at the same time are exposed to a number of environmental chemicals (Lewis et al., 2015; Zhang et al., 2014b). In this context, interactions among compounds may affect not only their efficacy (*i.e.*, medicinal drugs) but also their toxicity to the mother and the fetus (Rubinchik-Stern and Eyal, 2012). The placenta metabolizes numerous foreign chemicals such as environmental pollutants (*e.g.* polycyclic aromatic hydrocarbons) and medicinal drugs (*e.g.* codeine) (Myllynen et al., 2007; Pasanen and Pelkonen, 1990), less is known about trophoblast metabolizing capabilities. Here we demonstrate that trophoblasts may accomplish a crucial role in placental chemical metabolism and also that OP by CES regulation may further interfere with drug metabolization or activation.

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