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Characterization of placental cholinesterases and activity induction associated to environmental organophosphate exposure

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ABSTRACT

Although non-innervated, the placenta contains both cholinesterases (ChEs), butyrylcholinesterase (BChE) and acetylcholinesterase (AChE). These enzymes are well-known for their multiple molecular forms. In a first approach, we used recognized specific inhibitors, substrate preferences and non-denaturating gel electrophoresis in order to characterize the ChE profile of term placenta from uncomplicated pregnancy. Results strongly suggest that the predominant cholinesterasic form present was tetrameric BChE.

It is well established that both ChEs are targets of cholinesterase-inhibiting organophosphates (OP), one of the most important classes of chemicals actively applied to the environment. However, we have previously reported increased ChEs activity in placenta of rural residents exposed to OP. In the present work, we have studied: 1) whether this finding was reproducible and, 2) whether AChE or BChE up regulation is behind the increase of placental ChE activity. The population studied included forty healthy women who live in an agricultural area. Samples were collected during both the OP pulverization period (PP) and the recess period (RP). The placental ChEs activity increased in PP, evidencing reproducibility of previous results. The analysis of non-denaturating gels revealed that increased activity of total ChE activity in placenta from women exposed to OP may be attributable to tetrameric BChE up-regulation.

1. Introduction

Humans have two cholinesterases (ChEs): acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8). The enzymatic functions of both enzymes include hydrolysis of acetylcholine ACh [1]. At the nerve synapses, AChE terminates nerve impulse transmission by hydrolyzing this neurotransmitter. On the other hand, BChE acts as a backup for AChE and as a scavenger for poisons that might inhibit AChE activity [2]. These enzymes have been very rapidly distinguished and subject of considerable research [3].

Abbreviations: ChEs, cholinesterases; BChE, butyrylcholinesterase; AChE, acetylcholinesterase; ChE, cholinesterase; OP, organophosphates; PP, pulverization period; RP, recess period; ACh, acetylcholine; ASCh, acetylthiocholine iodide; BSCh, butyrylthiocholine iodide; iso-OMPA, tetraisopropylpyrophosphoramide; BW284C51, 1,5-bis (4-allyldimethyl ammoniumphenyl)-pentan-3-one dibromide.

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AChE and BChE are well-known for their multiple molecular forms [4]. Polymorphism is achieved by certain combinations of alternative gene splicing, and by the attachment of non-catalytic structural subunits. In mammals, AChE is encoded by a single gene. However, alternative splicing at the C-terminus of AChE mRNA generates three different isoforms. Conversely, one BChE transcript has been identified so far [5].

The presence of ChEs in tissues that are not cholinergically innervated provides the most compelling evidence that both AChE and BChE might have functions, other than the termination of cholinergic neurotransmission [6]. In fact, the human placenta contains an active cholinergic system which was associated to the amino acid uptake, the release of human placental chorionic somatotropin and prostaglandin production [7] and to the modulation of nitric oxide effect [8].

The concentrations of AChE and BChE are considerably lower in the placenta than in the nervous system [9]. The analysis by electron microscopy of cross sections from term placenta, cytochemically stained for ChEs activities, showed that term placenta syncytiotrophoblast cells produce primarily AChE. On the other hand, epithelial cells that surround the inner part of blood vessels, as well as hematopoietic cells present in them, all intensely stained for both AChE and BChE activities [10]. In accordance with these observations, it was reported that both AChE and BChE activities were detectable in cultured explanted villous of term placenta [11].

Depending on the experimental conditions used, dissimilar OP effects on placental AChE activity have been reported. Gestational exposure of rats to oral doses of the OP chlorpyrifos cause no inhibition of AChE activity [12], while a single cutaneous dose of OP in pregnant rats decreased AChE activity [13]. Nevertheless, we previously reported increased ChE activity in human placenta associated to OP environmental exposure [14].

Considering that AChE up regulation was induced post OP-treatment in rodents brain [3,15], muscle [16] and plasma [17], we speculate that an adaptive change could explain our previous finding.

The objective of the present study was to verify the reproducibility of the increased placental ChE activity associated to OP environmental exposure and to determine whether AChE up regulation is behind this finding. In addition, we also characterized placental ChEs activity in control samples using recognized specific inhibitors.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine (ASCh) iodide, butyrylthiocholine (BSCh) iodide, 5,5′-dithio-bis (c-nitrobenzoic acid) (DTNB), eserine hemisulfate salt, tetraisopropylpyrophosphoramide (iso-OMPA), 1,5-bis (4-allyldimethyl ammonium-phenyl)-pentan-3-one dibromide (BW284C51), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), EDTA (etilen diamino-tetracetic acid), boric acid, bovine serum albumin, Tris, glycerol, bromophenol blue, maleic acid, sodium citrate dihydrate, copper pentahydrate, potassium ferricyanide, cholinesterase acetyl (True Cholinesterase EC 3.1.1.7) Type V-S from Electric Eel, acrylamide, tetramethylenediamine and ammonium persulfate were purchased from SIGMA. Sodium dodecyl sulphate (99% pure) and ethanol (99% pure) were purchased from MERCK (Germany). Bisacrylamide was acquired from PROMEGA.

2.2. Participant recruitment and collection of samples

We performed a study of 40 healthy women ranging between 15 and 36 years of age incoming to prenatal care at the Cinco Saltos Public Hospital (Río Negro Province, Argentina), between December 2006 and August 2008. They were asked by a physician to participate in the study during their third trimester of pregnancy and, informed consent was obtained from each participant before they were interviewed. This study was carried out with the full ethical approval of the local Advisory Committee of Biomedical Research in Humans. The patients included in this study were residents of farms or communities surrounding fruit cultivation areas where pesticides, such as the OPs azinphos methyl, phosmet, chlorpyrifos and dimethoate, are applied during the spring and summer (September to February). Pesticides are usually finely dispersed as droplets at the time of pulverization and aerial drift from the target area is frequently, increasing the potential environmental exposure of the population. Samples collected from September to December were considered samples from the PP, and those collected from April to August were considered samples from the non-pulverization period or recess period (RP). A questionnaire was administered to document physical characteristics, educational level and lifestyle habits. Women with chronic diseases, on long-term medication (except those included in Group A according to the FDA), and those with serious pregnancy complications were excluded. Groups were matched for reported smoking habit and alcohol consumption. Placental villous samples were collected within 20 min of vaginal delivery. Suitable amounts from the central area of the maternal side of the placenta were obtained as the expression of various components that might vary according to the location. Samples were frozen at −20 °C until use.

In addition, placentas from urban residents with no history of pesticide exposure were collected during July–August 2006 to characterize placental ChEs activity. Similar exclusion criteria as those of the population study were used. Also, the full the local Advisory Committee of Biomedical Research in Humans approved this part of the study.

2.3. Cholinesterases activity and characterization

Small pieces of the tissue were cut and repeatedly washed with physiological solution and homogenized in ice-cold buffer. Then homogenates were filtered through a muslin cloth and centrifuged at 4 °C during 5 min at 4000 × *g*. AChE and BChE activities were determined in the supernatant according to the method of Ellman et al. [18]. In a typical assay, 2.6 ml of 0.1 M phosphate buffer pH 8, 100 μl of 0.01 M DTNB and 400 μl of the

sample supernatant were successively added in a standard cuvette. Measurement of enzyme activity was initiated by the addition of 20 μl of freshly prepared 75 mM ASCh iodide solution in distilled water. Absorption of the 2-nitro-5-thiobenzoate anion, formed from the reaction, was recorded at 412 nm for 2 min at 30 °C. Spontaneous substrate hydrolysis was assessed using a blank without sample. Kinetic was calculated in the linear range. Each sample was analyzed by triplicates. Protein concentration was determined according to Lowry et al. [19].

The enzymatic activity was expressed as μmol of substrate hydrolyzed per minute per mg of protein, using a molar extinction coefficient of 1.36×10^{-3} M^{-1} cm^{-1}.

The characterization of ChE was carried out using the following substrates: ASCh (considered non-selective) and BSCh (specific for BChE). Substrate concentrations varied from 37.5 to 150 mM (final concentrations in the cuvette: 0.24–0.96 mM). In the selective inhibitor experiments, all enzymatic activities were determined using ASCh as substrate at the 75.0 mM concentration (final concentration in the cuvette: 0.48 mM). The following inhibitors were used: eserine sulphate, BW284C51 and iso-OMPA, which selectively inhibit total ChEs, AChE, and BChE, respectively. Final inhibitor concentrations were 1.25–25 μM for eserine, 0.85–13.20 μM for BW284C51, and 1.00–64.00 μM for iso-OMPA. Stock solutions of eserine and BW284C51 were prepared in water, and iso-OMPA stock solution was dissolved in ethanol. Each inhibitor solution (5 μL) was mixed with 495 μL of the homogenate and incubated at room temperature for 20 min as described by Nunes et al. [20]. Water was used as a control, and an additional control was prepared with ethanol for the samples exposed to iso-OMPA. All the experiments were performed in triplicate.

2.4. Gradient gel electrophoresis

A total of 0.12 g of placenta, containing about 0.9 mg protein, were cut in small pieces, repeatedly washed with physiological solution and homogenized on ice with 1.5 M Tris–HCl buffer pH 8.8. Then, the homogenate was filtered through a muslin cloth and centrifuged at 4 °C during 5 min at $4000 \times g$. The supernatant was mixed with an equal volume of 50% glycerol, 0.1 M Tris–HCl, pH 7.5 and bromophenol blue before loading on the gel with the sample containing 10 μg of protein [19] per line. Four to thirty percent polyacrylamide gels, 0.75 mm were poured in a Hoefer gel apparatus [21]. Electrophoresis was run at 250 V for 20 h at 4 °C.

For the population study, fourteen samples (PP) and ten samples (RP) chosen at random in each group were processed as describe above.

The study population for electrophoresis analysis consisted of fourteen samples (PP) and ten samples (RP) chosen at random in each group.

2.5. Staining for AChE and BChE activities

The method of Karnovsky [22] adapted to polyacrylamide gels by Li et al. [21] was used. The staining solution contained 180 ml of 0.2 M maleic acid adjusted to pH 6.0 just before use, 15 ml of 0.10 M sodium citrate, 30 ml of 0.030 M $CuSO_4$, 30 ml water, 30 ml of 5 mM potassium ferricyanide, and 150 mg of ASCh iodide or 171 mg of BSCh iodide. AChE purified from electric organ tissue of *Electrophorus electricus* was used as a staining technique control. Gels were incubated for 5 h, or overnight, with gentle shaking until brown-red bands of activity developed.

2.6. Identification of ChEs bands on gels

Gels stained with ASCh revealed both AChE and BChE because both enzymes have high activity with ASCh. On the other hand, gels stained with BSCh identified BChE. In addition, comparison with human serum bands allowed the identification of BChE according to the attachment of structural subunits.

2.7. Data analysis

An analysis of variance (ANOVA) was performed to compare differences between the inhibitor and the substrate concentrations. The comparison between the two types of substrates at same concentration and between PR and PP samples was made using the Students *t*-test. All statistical analyses were performed using R software version 2.6.0. Statistical significance was assumed as $p < 0.05$.

3. Results

3.1. Characterization of placental ChEs in control samples

The characterization of the ChEs activities included a first step to distinguish ChEs from non-specific esterases using *in vitro* incubations with specific inhibitors of ChEs. Fig. 1A shows that ChEs activities were almost totally inhibited by eserine. Specific inhibitors were used, an important decrease of BChE and AChE enzymatic inhibition was observed, reaching a plateau at about at 3.3×10^{-6} M (Fig. 1B) and 16×10^{-6} M (Fig. 1C), respectively.

The preference of placenta homogenate samples using ASCh or BSCh as substrates is shown in Fig. 2. Lower enzymatic activities were observed when using BSCh as substrate at all the evaluated concentrations (ASCh > BSCh ($p < 0.05$)).

3.2. Identification of a unique placental ChE band on gels from control samples

Fig. 3 shows the migration of the bands corresponding to control placenta samples, human plasma sample and commercial *E. electricus* AChE. Enzyme activities were revealed in the presence of the BChE-specific substrate, BSCh (Fig. 3A) and in the presence of ASCh (Fig. 3B). In both gels, monomer, dimer, and tetramer bands of serum BChE were revealed; while a unique band at the same location of BChE plasma tetramer, was stained in placenta samples.

3.3. Population study

The characteristics of the study groups as well as the blood ChE activities were reported previously [23]. Briefly,

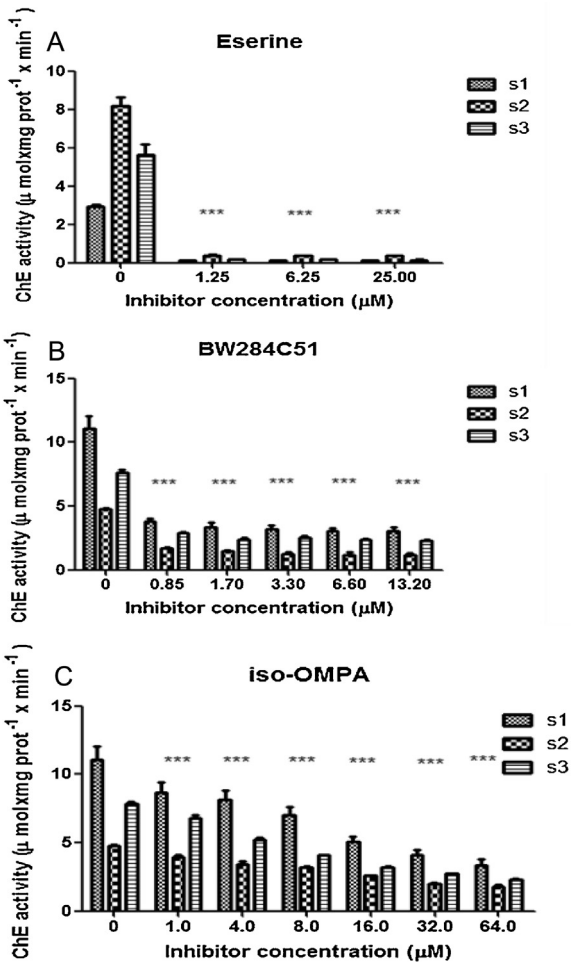

Fig. 1. Effect of specific inhibitors of cholinesterase activity. Activity was determined by the Ellman assay using ASCh 0.48 mM as substrate. Results are presented as means ± SD of three placenta samples (s1, s2 and s3) in triplicates each of them. ANOVA: $^{***}p < 0.0001$.

PP and PR groups were similar in terms of demographical characteristics and habits. Only 1.2% (RP) reported alcohol consumption (less than two alcoholic beverages/week) and 5% and 6.2% (RP and PP, respectively) had smoked during

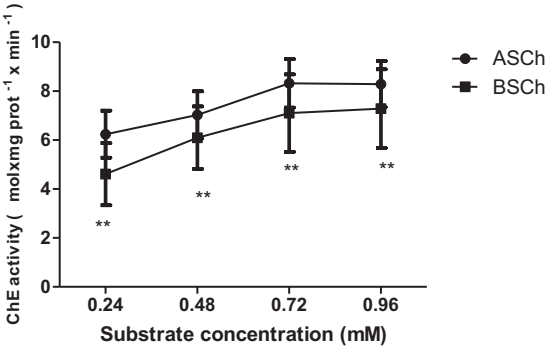

Fig. 2. Substrate preference of cholinesterases from total placenta homogenates. Results are presented as means ± SD of three placenta samples in triplicates each of them. ASCh *vs.* BSCh: *t*-test $^{**}p < 0.05$.

Table 1
The results were expressed as mean ± SD.

	Cholinesterase activity
RP ($n = 18$)	9.28 ± 4.99
PP ($n = 22$)	16.34 ± 5.51**

Cholinesterase activity in placenta homogenate was expressed as µmol of substrate hydrolyzed/mg protein^{-1} min^{-1}. Activity was determined by the Ellman assay using ASCh 0.48 mM as substrate.
** $p < 0.001$, *t*-test.

pregnancy. Comparing the average blood ChE activity of RP *vs.* PP, plasma BChE decreased significantly (20%, $p < 0.01$), suggesting maternal anticholinesterase pesticide exposure in PP.

3.4. Environmental OP exposition increased placental ChE activity

As shown in Table 1, placental ChE activity was affected by the sampling period. The average ChE activity of placental homogenates increased significantly 76% ($p < 0.001$) in PP.

3.5. Environmental OP exposition increased BChE band

A representative gel of placenta samples from RP and PP groups is shown in Fig. 4. The comparison of RP sample

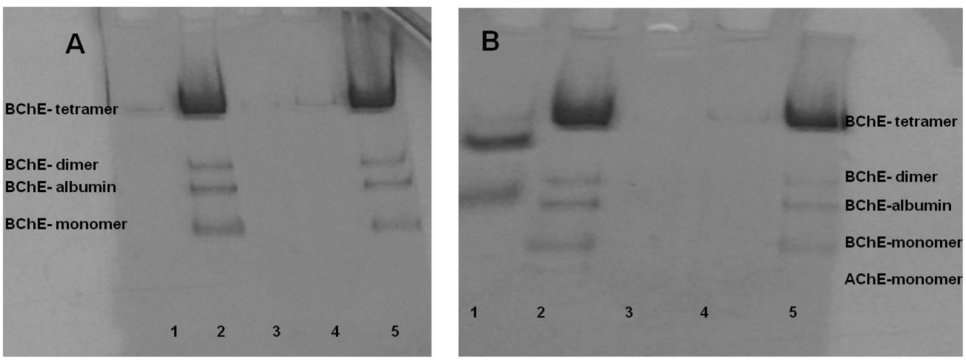

Fig. 3. Non-denaturing gel stained for activity with BSCh (A) and ASCh(B).
(A) and (B) line 1, AChE Electric Eel. Lines 2 and 5, human plasma samples. Lines 3 and 4, placenta homogenate samples. The band labeled BChE-alb is a disulfide-bonded dimer between one subunit of BChE and one molecule of albumin.

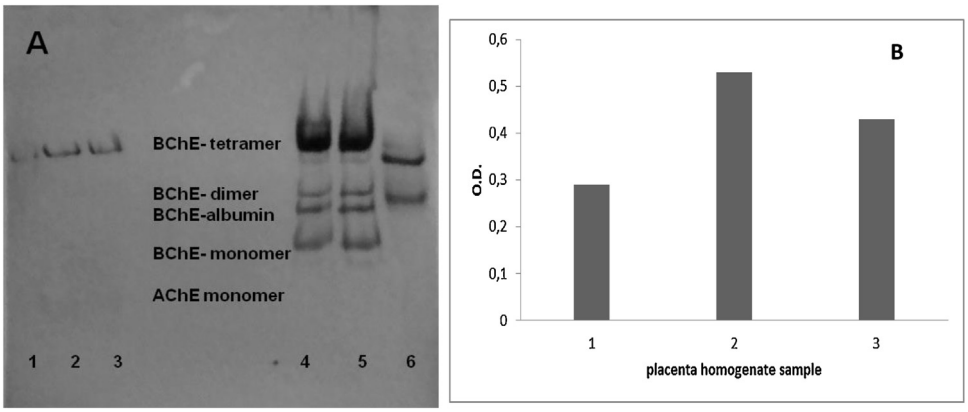

Fig. 4. (A) Non-denaturing gel stained for activity with ASCh. Line 1, placenta homogenate sample (RP). Lines 2 and 3 homogenate samples (PP). Lines 4 and 5 human plasma samples and line 6 AChE Electric Eel. (B) The bar graph represents the densitometry quantification of protein levels in the placenta homogenates. Values obtained for the optical densities were: 0.29; 0.53 and 0.43.

(line 1) and PP samples (lines 2 and 3) demonstrated a higher intense band in RP sample and suggest the same location of BChE plasma tetramer.

4. Discussion

4.1. Placental ChE characterization

As expected, in the present study the measured enzymatic activity in placenta homogenates was almost fully inhibited by eserine hemisulfate (Fig. 1A). The results observed with this generic inhibitor of ChE, confirmed previous reports. Our results, are also in consonance with those of Fant and Harbison [24] and Derewlany et al. [25] who previously showed activity on both ChE from different subcellular fractions of placenta. Inhibitors incubations showed that one of them presents the properties of a vertebrate BChE: high sensitivity to eserine hemisulfate (Fig. 1A) and iso-OMPA (Fig. 1C). It must be noted that the incubation with the chemical iso-OMPA, specific inhibitor of BChE, resulted in significant but non complete inhibition. This result suggested that the remaining activity reflects the relative contribution of AChE to ASCh hydrolysis. In fact, there was another which presents all the properties of a vertebrate AChE: high sensitivity to eserine and BW284c51 (Fig. 1B). Also, a partial sensitivity to BW284c51 was observed for AChE.

As stated, enzymatic activity using ASCh represents combined AChE and BChE activities. Therefore, the substrates preference of placenta homogenate samples suggests that BChE contributed almost to the 75% to the total ChE hydrolysis of ASCh (Fig. 2). In accordance with the substrates preference assay, the non-denaturing gradient gel electrophoresis of placenta samples revealed only one band when stained with both AChE (Fig. 3A) and BChE (Fig. 3B), showing that BChE activity represents total placental ChEs activity detected by this method. In agreement, the content of ChE mRNAs by RT-PCR in human kidney samples, showed that this organ possesses abundant BChE activity and less AChE activity in the form of GPI-anchored species [26]. Also, it was reported that AChE activity in mice predominates only in the brain while BChE activity is higher in intestine, serum, lung, liver, and heart [27].

Comparison with recognized bands of plasma BChE isoforms [21], the band position strongly suggests that tetrameric BChE is the predominant ChE form in placental homogenate. It was reported that BChE can occurs as various globular forms (G1, G2 and G4), as amphiphilic or hydrophilic variants. The latest form is abundant for BChE in mammalian body fluids and in the soluble fraction of tissue homogenates [28].

Our results differ from that of Hahn et al. [11] who found higher AChE activity than BChE activity in cultured explanted villous of term placenta. It must be noted that although placental explant cultures are useful for studying tissue functions, experimental conditions such as concentrations of ions and nutrients in culture media can have important implications for villous function [29]. In addition, Hahn et al. [11] used a higher speed supernatant (16,000 rpm) as a enzymatic source than the one from the present study (5,700 rpm).

In summary, we propose that in human placenta homogenates, ChE activity mostly corresponds to BChE on the basis of its inhibition with iso-OMPA, substrate preferences and non-denaturating gel electrophoresis mobility. Minor AChE activity was also detected.

In the current work, placental ChE increased in PP respect to RP samples, reproducing our previous finding in placenta of women living in the same area [14]. In addition, the comparative analysis of the position and intensity of a unique band in non-denaturing gel of PP and RP samples suggests that BChE tetramer is behind the increase in placental ChE activity. Instead, we previously reported lower blood BChE activity associated to PP in the participants included in the present study [23].

Using the same analytical method, Duysen et al. [30] showed trimers or dimers of AChE in plasma of mice intoxicated with different OPs. Mice treated with OP responded with the classic signs of OP exposure. In the first hours and days after OP treatment, plasma AChE and BChE activities were inhibited. Over the next few days, both activities were recovered. After that, the AChE activity was increased to

2.5-fold above of the normal activity. Herein, we demonstrated for the first time that OP environmental exposure is associated to BChE up regulation in a non-innervated tissue. In summary, the current study strongly suggest that an up regulation of placental BChE, recognized as the first line of defense against poisons and drugs [31] is associated to environmental OP exposure. We propose that it represents an adaptive change in BChE gene expression as mediator of recovery from chemical OP insults. As it appears that BChE has two principal functions: detoxification and acetylcholine hydrolysis [32], it remains to be elucidated whether this response might affect cholinergic system function in placenta.

Transparency document

The Transparency document associated with this article can be found in the online version.

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