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PROTEIN ADDUCTS AS BIOMAKERS OF EXPOSURE TO ORGANOPHOSPHORUS COMPOUNDS

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Abstract

Exposure to organophosphorus (OP) compounds can lead to serious neurological damage or death. Following bioactivation by the liver cytochromes P450, the OP metabolites produced are potent inhibitors of serine active-site enzymes including esterases, proteases and lipases. OPs may form adducts on other cellular proteins. Blood cholinesterases (ChEs) have long served as biomarkers of OP exposure in humans. However, the enzymatic assays used for biomonitoring OP exposures have several drawbacks. A more useful approach will focus on multiple biomarkers and avoid problems with the enzymatic activity assays. OP inhibitory effects result from a covalent bond with the active-site serine of the target enzymes. The serine OP adducts become irreversible following a process referred to as aging where one alkyl group dissociates over variable lengths of time depending on the OP adduct. The OP-adducted enzyme then remains in circulation until it is degraded, allowing for a longer window of detection compared with direct analysis of OPs or their metabolites. Mass spectrometry (MS) provides a very sensitive method for identification of post-translational protein modifications. MS analyses of the percentage adduction of the active-site serine of biomarker proteins such as ChEs will eliminate the need for basal activity levels of the individual and will provide for a more accurate determination of OP exposure. MS analysis of biomarker proteins also provides information about the OP that has caused inhibition. Other useful biomarker proteins include other serine hydrolases, albumin, tubulin and transferrin.

Keywords

Biomarkers; Organophosphates; Protein adducts; Mass spectrometry

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1. Introduction and background

Humans can be subjected to an array of chemicals present in the environment, either by occupational, accidental or intentional exposure. Organophosphates (OP) are toxic compounds that include insecticides and chemical warfare agents, as well as antiwear agents and naturally occurring OP toxins. OPs were discovered in the mid-19th century, but their use in agriculture began following the World War II (Nigg and Knaak, 2000; Petroianu, 2010). OP insecticides, including azinphos methyl, chlorpyrifos and malathion, became widely used in agriculture for the control of insect pests in the 1970s, when the environmentally persistent organochlorine pesticides were banned. Their use in the US has decreased by more than 60% since 1990, from 85 million pounds in 1990 to 33 million pounds in 2007, with chlorpyrifos, malathion and acephate being the most used [US Environmental Protection Agency (US EPA) 2007]. OPs are also used in residential settings and other non-agricultural sectors, but to a much lower extent.

Although OPs insecticides are less inexpensive, less persistent in the environment and less susceptible to pest resistance, they are more toxic to mammals than organochlorines. Their primary mechanism of action is the inhibition of cholinesterases and other serine active-site enzymes, causing a variety of adverse health effects. OP insecticides are involved in many occupational poisoning cases, although accidental exposures in residential settings or environmental contaminations also occur (Bolognesi, 2003). As a consequence, human biological monitoring of these exposures has become essential. The term “biomonitoring” was originally defined as “a systematic standardized collection of biological samples for the measurement of a substance or its metabolites in body fluids (including blood and urine) of exposed subjects with the objective to assess exposure and health risk” (Angerer et al. 2007; Kapka-Skrzypczak et al. 2011). Biomonitoring of exposures involves the measurement of a substance termed biomarker that can identify, track or monitor the state of the exposure and other aspects of health (Kim et al. 2010). Biomarker identification and characterization is critical for risk assessment and is an expanding area of research.

This review will focus on the detection of OP-adducted protein biomarkers as a more sensitive and accurate assessment of OP exposure.

2. Biological effects of OP exposure

Biological effects of OP exposure include headaches, dizziness, salivation, lacrimation, diarrhea, tremors, bradycardia, convulsions, coma and even death (Costa, 2006; Jokanovic et al. 2011). These symptoms are a result of acetylcholine accumulation in synapses, causing overstimulation. Thus, acetylcholinesterase, the enzyme that hydrolyzes acetylcholine, is the main target for OPs. Other serine hydrolases are also inhibited by OPs (Casida and Quistad, 2004). Some OP compounds have been shown to induce a delayed neuropathy, known as OP-induced delayed neuropathy (OPIDN), which manifests 1–3 weeks after exposure. OPIDN is characterized by axonal degeneration of central and nervous peripheral systems (Lotti and Moretto, 1999). OPIDN appears to result from inhibition and aging of a lipase/esterase known as neuropathy target esterase (NTE) present in nerve tissue (Casida et al. 2008; Glynn, 1999; Johnson, 1969). The dose and duration of the exposure, as well as the nature of the OP are factors that have been reported to modulate the effects induced by OP exposure (Pope, 1999).

The inhibition of serine hydrolases by OPs is due to the formation of a covalent bond between the alkyl phosphorus group of the OP and the hydroxyl residue of the active-site serine. Although this phosphorylation is initially reversible, with time this complex can undergo further metabolic reactions, known as “aging” (Masson et al. 2010). The aging process consists in the loss of one of the two alkoxy groups of the OP attached to the

phosphorus atom (Nigg and Knaak, 2000). Some OP adducts undergo further aging (e.g. cresylphosphoserine), leaving a phosphate attached to the active-site serine (Fig.1) (Schopfer et al. 2010). Once the adduct has aged, the binding to the active-site serine is permanent.

The current treatment for OP exposures consists of the administration of the drug atropine, which competes with acetylcholine for binding to muscarinic receptors at the synapses, decreasing the effect of neurotransmitter accumulation. Atropine is administered in combination with oximes, pralidoxime being the most commonly used. Oximes are nucleophylic compounds that can reactivate cholinesterases before they undergo aging. The efficacy of oximes in reactivation depends on the specific enzyme and the chemical structure of the OP compound bound to the enzyme. It also depends on the delay in the administration of the treatment. Some OP adducts such as the one generated by the nerve agent soman age very rapidly. There is some controversy as to whether oximes are beneficial as a first treatment for exposed subjects (Barelli et al. 2011; Eyer, 2003). For instance, a randomized double-blind placebo-control trial published in 2009 concluded that pralidoxime administration did not improve survival or reduce the need for intubation in subjects with OP poisoning (Eddleston et al. 2009).

3. Biomonitoring OP exposures

Biological monitoring consists of assessing exposure and health risk in a workplace, compared with a non-risk environment (Maroni et al. 2000). In 1949, after an agricultural worker was killed by an acute exposure to parathion in Florida (United States), the Florida citrus industry began biological monitoring of the workers (Griffiths et al. 1951; Nigg and Knaak, 2000). Their purpose was to establish directions for the use of parathion by correlating exposures to parathion with changes in blood cholinesterase levels. Currently, Florida does not require cholinesterase monitoring of agricultural workers, although the University of Florida has an ongoing cholinesterase monitoring program in which workers can voluntarily enroll in it.

Originally, attention on biomonitoring OP exposures was focused on the cholinesterases (ChEs) AChE and butyrylcholinesterase (BChE), the first OP targets described. OPs are irreversible inhibitors of both ChEs, meaning that only new enzyme synthesis will restore activity (Gutmann and Besser, 1990; Koelle and Gilman, 1946). Inhibition of blood ChEs have long served to assess the physiological course of OP exposures, especially plasma BChE activity since it is more sensitive to inhibition by some OPs than red blood cell (RBC) AChE, and also recovers more rapidly following exposure. Inhibition of RBC AChE is a good marker of neuronal AChE inhibition, and therefore, a good marker of the severity of the OP intoxication. Taking into account that a 50% inhibition of neural AChE is necessary for developing anti-cholinergic symptoms, early detection of exposures by monitoring blood ChEs can allow interventions before severe symptoms occur. In addition, these measurements also enable health care providers to follow recovery of the subject.

The standard method in use for biomonitoring OP exposures is the Ellman colorimetric enzymatic assay, which uses acetylthiocholine to determine AChE activity or butyrylthiocholine to determine BChE activity (Ellman et al. 1961). These assays are accurate, reliable and inexpensive. Two other methods are also used to monitor ChE activities, the end point delta pH method of Michel, which measures acetylcholine breakdown measuringchanges in the pH, and the radioactive method of Johnson and Russell, which extracts radiolabeled acetylcholine from its hydrolysis products (Wilson et al. 2005). The data from these assays are not necessarily inter-convertible, and because the pH method is slow, and the radioactive assay is expensive and uses radio isotopes, the Ellman assays

have been the methods preferred. Unfortunately, these assays present some drawbacks. First of all, pre-exposure activity determinations are required (Cocker et al. 2002; Garabrant et al. 2009). It is often difficult to obtain accurate baseline measurements for exposed individuals, and there are some variations in activities of ChEs over time. These are not only interpersonal variations, but also intrapersonal as a result of biological variability, certain diseases and medications that may affect ChE levels (Lepage et al. 1985; Nigg and Knaak, 2000). Furthermore, the Ellman assays are not accurate at exposures causing 20% or less ChE inhibition, and cannot elucidate which class of OP caused the inhibition (Sun and Lynn, 2007). In addition, the Ellman assays are not suitable for biomonitoring the full range of OP exposures, since some OPs can cause adverse health effects with low levels of ChE inhibition (Casida and Quistad, 2004; Pope, 1999). Despite these drawbacks, the Ellman assays are currently the standard method for monitoring OP exposures, including agricultural worker exposures in the states of California and Washington in the United States.

An alternative approach involves the analysis of the dialkyl phosphate (DAP) and leaving group metabolites in urine. In the late 1960s, Shafik and Enos initiated these analyses (Shafik and Enos, 1969). DAP metabolites are produced during the hepatic and serum metabolism of OPs, through bioactivation and/or detoxification pathways, and are eliminated in the urine. A leaving group, specific for each OP, is also generated during this metabolic process. Neither DAPs nor the leaving group are cholinesterase inhibitors. Thus, the disadvantage of urinary DAP analysis is that they do not provide information about the OP of exposure, since the metabolism of a given OP can release different DAPs, and same DAPs can be released by different OPs (Sudakin and Stone, 2011). In addition, DAPs and the leaving group are not only produced *in vivo*, it is widely known that they can be detected in the environment. Once OPs are applied, they undergo degradation by a variety of processes, including hydrolysis, photolysis and microbial degradation (Racke, 1992). There are studies demonstrating the presence of DAPs or trichloropyridine (the leaving group specific for chlorpyrifos hydrolysis) in food as well as contaminated surfaces (Eaton et al. 2008; Morgan et al. 2005; Sudakin and Stone, 2011). Thus, detection of urinary DAPs or the corresponding leaving group cannot discriminate between OP exposure and direct environmental exposure to non-toxic OP degradation products, resulting in possible overestimation of the exposure (Egeghy et al. 2011; Morgan et al. 2011). Furthermore, their half-lives are short, ranging between 24 and 48 h following exposure. On the other hand, measurement of these urinary metabolites is less invasive and logistically simpler than the ChE activity monitoring (Bravo et al. 2004; Cocker et al. 2002), providing incentive for use of these analyses. Further studies are required to establish the relevance of urinary metabolite analysis in assessing OP exposures. Nonetheless, DAP metabolites are common for the majority of OP compounds, and while blood test for detecting OP exposures are still under development, measurement of DAPs provides valuable information about chronic OP exposures (Barr and Angerer, 2006; Barr et al. 2004).

4. Identification and characterization of protein biomarkers of OP exposure

There is increasing interest in the analysis of protein biomarkers of OP exposure since the OP adducted proteins have longer half-lives than parent compounds or metabolites. Proteins are modified only by the active pesticide or nerve agent and their breakdown products will not react with target biomarker proteins. Proteins from the blood compartment fit the criteria for useful biomarkers: they are biological proteins that can be analyzed to provide key information about the health of the subject and the origin of the exposure, they can be analyzed, they have relatively long half-lives, they are also relatively inexpensive to assay, and they provide unequivocal information about the nature and level of OP exposure (Costa,

1996; Henderson et al. 1989; Kim et al. 2010). Various blood proteins serve as biomarkers of exposure, effect or susceptibility. This review focuses on biomarkers of OP exposure.

Serine hydrolases, especially ChEs, are classical biomarkers that have been studied and monitored primarily because they contain a serine in the active site, and historically it was thought that they were the only targets for OP compounds. However, it has been observed that chronic low levels of certain OPs can cause neurotoxicity without significant ChE inhibition, indicating that other proteins are also important OP targets (Ray and Richards, 2001; Richards et al. 1999). These other biomarker proteins may well serve as more sensitive biomarkers than ChEs for some OPs.

The Ellman assays, the conventional method for monitoring OP exposures, are focused on determining the remaining uninhibited cholinesterase activity. Unfortunately, as described in the previous section, this method has several drawbacks. Thus, other approaches have been developed, based on the detection of the inhibited enzyme. One of them involved the use of fluoride ions to reactivate the inhibited enzyme. In this process, the original phosphofluorides are regenerated and can be identified and quantified by gas chromatography coupled with mass spectrometry (GC-MS) (Polhuijs et al. 1997). This method provides identification of both the OP and extent of the exposure. However, development of this approach is limited by the impossibility of reactivating aged phosphorylated enzymes.

Following the development of approaches for monitoring OP-adducted enzymes, liquid chromatography coupled with MS (LC-MS) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS have become important tools for biomonitoring OP-adducted enzymes (John et al. 2008). They allow for the accurate and sensitive identification of OP-inhibited proteins based on the change in mass of the adducted active-site peptides following enzymatic digestion. Since serine esterases have a very stable adduct on their active-site serines following the aging process, analysis by LC-MS or MALDI-TOF can identify and characterize adducts by detecting the difference in the mass of the active-site peptide. These OP-adducted enzymes remain in circulation for times that are dependent on their half-lives, which are generally much longer than the half-lives of the parent OP compound or its metabolites, providing a longer window for detection than the analysis of urinary metabolites or free OPs in blood or urine. Further, it is possible to learn something about the OP of exposure due to the mass differences between different adducts.

The proteomics techniques have also proved to be useful for the identification of new biomarkers of OP exposure (Thompson et al. 2010).

4.1. Acetylcholinesterase and Butyrylcholinesterase

AChE (referred in the past as “true cholinesterase”, EC 3.1.1.7, accession # P22303), is a tetrameric serine esterase glycoprotein, composed of 70 kDa subunits, and is found in the terminal synapses of central and peripheral nerves, where it terminates synaptic transmissions via hydrolysis of the neurotransmitter acetylcholine. As noted before, this enzyme is also found on RBC membranes. AChE has been shown to be the primary target of OP toxicants in mammals and insects (Koelle and Gilman, 1946).

BChE (also known as pseudocholinesterase or plasma cholinesterase, EC 3.1.1.8, accession # P06276), is also a serine esterase glycoprotein, composed of four 85-kDa subunits, and is present at high levels almost ubiquitously, but primarily in glial cells, liver and plasma. BChE also hydrolyzes acetylcholine, as well as many other esters, including synthetic and naturally occurring poisons. Therefore, it can act as a backup for AChE, as demonstrated with the AChE knockout mouse (Duysen et al. 2001), even though acetylcholine hydrolysis

is not the primary role of BChE. However, the physiological function of this enzyme is still unclear.

The human body has approximately10 times more BChE than AChE (Manoharan et al. 2007). In vertebrates, both ChEs are inhibited by the alkaloid eserine, a property that distinguishes them from non-specific esterases (Chatonnet and Lockridge, 1989). They exist as soluble or membrane-bound polymers of catalytic subunits, with each subunit containing an active-site serine (Nigg and Knaak, 2000). The most notable differences between these two B-esterases are their substrate specificities, acetylcholine *versus* butyrylcholine, their inhibition (AChE) or not (BChE) by excess substrate, and their expression during tissue differentiation and development (Chatonnet and Lockridge, 1989). In addition, they show different sensitivities for inhibition by different OP compounds (Aldridge, 1953).

The first compound known to inhibit ChEs, tetraethyl pyrophosphate, was synthesized in 1855 by Philipe de Clermont (Petroianu, 2010). Almost a century later, Langer and Krueger synthesized alkyl fluorophosphate esters and described their toxicity, which led to an increasing interest in OPs. These were the first OP compounds known to irreversibly inhibit ChEs (Mackworth and Webb, 1948; Mazur and Bodansky, 1946). The most potent member of this group, di-isopropyl fluorophosphate (DFP), has been extensively studied. *In vitro* and *in vivo* studies on the mechanism of ChE inhibition by DFP revealed that once the OP binds covalently to either ChE, the enzyme-inhibitor complex formed does not dissociate and the inhibition of activity persists for many days (Mazur and Bodansky, 1946). These authors also reported that in humans, plasma ChE was more sensitive to inhibition by DFP than RBC or brain ChE, and that they have differential rates of restoration of activity. Another interesting observation was that following low levels of DFP exposure, BChE was inhibited in the absence of cholinergic symptoms. In the study of the mechanism of ChE inhibition by OPs, an initial observation was that OPs and acetylcholine compete for binding to ChE, indicating that OPs bind to the same active center of the enzyme (Augustinsson and Nachmansohn, 1949). An observation by Aldridge was that different OPs have different affinities for the ChEs, probably related to the composition of the alkyl groups attached to the phosphorus, which modify their affinity for the ChEs, similar to the substrate preferences of acetylcholine and butyrylcholine (Aldridge, 1953).

As noted above, inhibition of blood ChE (RBC AChE or plasma BChE) activities is currently the standard method used for the biological monitoring of OP exposure. Thus, ChEs are useful biomarkers of OP exposure since they are very sensitive to inhibition by many OP compounds and are found in blood, a matrix that is easy to obtain and analyze. One of the advantages of the enzymatic measurement of AChE *versus* BChE as a biomarker of exposure is the half-life. RBC AChE has a 120 day life-span (≈33 day half-life; Mock et al. 1999), although once it is inhibited, it has been shown that the recovery of AChE activity occurs after approximately 80 days (Mason, 2000), suggesting that either exposed RBCs are eliminated faster than unexposed RBCs or that some of the OP-inhibited AChE may be reactivated. In contrast, BChE has an 11-day half-life in plasma (Mason, 2000; Ostergaard et al. 1988). Therefore, RBC AChE enables the evaluation of exposure for a longer period of time following exposure. On the other hand, the enzymatic measurement of BChE has some advantages over AChE. Starting with the availability, there is more BChE than AChE in blood. BChE is present as a soluble protein in plasma, while AChE is primarily found in the RBC membrane. When assaying RBC ChE, there are difficulties in reproducibly removing serum interferences from the RBCs and possible interference from the red cell hemoglobin during the assay (Wilson et al. 2005). BChE can hydrolyze or scavenge a broader range of OPs than AChE, probably due to differences in the geometries of their active-site gorges (Masson et al. 2010).

These drawbacks of the Ellman assay prompted research on MS-based assays focused on the identification of OP-adducted proteins. AChE and BChE have been the most studied and most used biomarkers for OP exposure, so they were the first examined by MS analysis. Doorn and co-workers were the first to detect adducts with pure equine serum BChE inhibited *in vitro* with isomalathion using MALDI-TOF (Doorn et al. 2001). A similar approach using recombinant human AChE inhibited with various OPs was published in the same year (Elhanany et al. 2001). However, as BChE is soluble and is abundant in plasma, analysis of BChE adducts proved to be more useful. A year later, another method was described that involved the use of liquid chromatography coupled with MS (LC/MS) to analyze *in vitro* and *in vivo* nerve agent-inhibited BChE (Fidder et al. 2002). They suggested that the same approach should work for OP pesticides and carbamates.

Following these first reports, a number of studies using similar approaches have been published. The major focus of these studies has been on BChE (Li et al. 2008a; Li et al. 2010a; Liyasova et al. 2011; Noort et al. 2006; Sun and Lynn, 2007; Thompson et al. 2010). The main limitation of these studies has been the difficulty in standardizing the method for high-throughput analyses of exposures. The critical step is the purification of BChE from plasma. All of the above studies used affinity chromatographic purifications that are time-consuming and require relatively large sample volumes and the use of special resins. In 2007, highly sensitive capture methods using antibody-coupled magnetic beads, coupled to LC-MS/MS were reported (Whiteaker et al. 2007). The use of immune-affinity purification of BChE, coupled with MS, should overcome these disadvantages (Aryal et al. 2012; Kim et al. 2010; Marsillach et al. 2011; Sporty et al. 2010). The rapid purification of BChE by immunomagnetic-bead separation (IMS) requires smaller quantities of plasma and can be easily automated. For immunoprecipitation protocols, it is important to identify a useful antibody and magnetic beads that provide reproducible purifications with low background. Furthermore, the IMS protocols are amenable for high-throughput robotic sample processing. In all of the MS studies, once BChE has been purified, it is digested to peptide fragments using proteolytic enzymes (trypsin, chymotrypsin, pepsin, etc.). The peptides are then analyzed by MS (Fig. 2). The adducted active-site peptide is identified using a database match.

4.2. Acylpeptide hydrolase

Acylpeptide hydrolase (APH, EC 3.4.19.1, accession # P13798) is a serine active-site esterase/protease that cleaves N-acetylated peptides and most likely plays an important role in intracellular protein turnover (Raphel et al. 1999). It is a tetrameric protein composed of 81-kDa subunits that is present in many tissues, including brain and liver. In circulation, it is found in the cytosol of RBCs, where its role appeares to be degradation of proteins modified by glycation or oxidation (Fujino et al. 2000). This enzyme becomes attached to the membrane when the erythrocyte is oxidized (Fujino et al. 1998).

Although in 1987 it was reported that DFP inhibited APH activity (Kobayashi and Smith, 1987), the first evidence of APH as a non-cholinergic target for OP compounds came in 2000 (Richards et al. 2000), when it was shown that APH was a direct target for some OPs and that it was more sensitive than AChE to OPs such as dichlorvos, both *in vitro* and *in vivo*. Some years later, it was demonstrated that murine RBC APH could serve as a potential biomarker of OP exposure (Quistad et al. 2005). In this study, it was shown that mouse APH sensitivity to OP exposure was dependent on the specific OP. For example, as noted, APH was more sensitive to dichlorvos compared with BChE or AChE; it was as sensitive as BChE to tribufos; however, it was not sensitive to 10 other OPs tested. In addition, APH inhibition could be detected after longer periods of time compared to BChE, an advantage resulting from the long half-life of RBCs. It has also been suggested that APH may be implicated in cognitive processes *via* a non-cholinergic pathway following exposure to OPs

(Pancetti et al. 2007; Richards et al. 2000). More recently, *in vitro* OP-adducted human RBC APH has been characterized by IMS followed by LC-MS/MS (Kim et al. 2010; Marsillach et al. 2011), supporting the use of APH as a biomarker for OP exposure (Quistad et al. 2005). These studies made use of *in vitro* inhibited samples; there are still no MS data on APH adducts from OP exposed individuals.

As mentioned, one of the advantages of using RBC APH as a biomarker of exposure is the long half-life of RBCs (Mock et al. 1999), and the slow recovery of the activity following exposure to OPs (Quistad et al. 2005). The 33 day half-life of RBCs allows for a longer window in time for measuring the percentage of RBC APH active-site serine adducted by OPs. One of the disadvantages is that APH in sensitive to some, but not all, OP compounds.

4.3. Albumin

Albumin, which has a 20 day half-life, is the most abundant protein found in plasma. It is a 67-kDa multifunctional monomer synthesized and secreted by the liver. Albumin possesses esterase-like and aryl acylamidase activities but it does not have a catalytic active-site serine. It is generally accepted that OPs cause irreversible inhibition of serine hydrolases by binding to their active-site serines. Nevertheless, some *in vitro* and *ex vivo* studies have already demonstrated covalent binding of OPs, such as soman and sarin, chlorpyrifos oxon, dichlorvos, DFP and parathion, to albumin (Black et al. 1999; Li et al. 2007; Mourik and de Jong, 1978; Sanger, 1963). Lockridge and co-workers were the first to detect OP adducts on albumin in mice (Peeples et al. 2005; Schopfer et al. 2005) and exposed humans (Li et al. 2010b). They proposed albumin as a useful biomarker for OP exposure. The administration of low doses of OPs to mice did not inhibit AChE activity but labeled albumin mainly on tyrosine-411, the most reactive residue of albumin (Means and Wu, 1979; Schopfer et al. 2005). The detection of adducted albumin was carried out with tagged irreversible inhibitors [fluorophosphonate (FP)-biotin] followed by MALDI-TOF analysis. The use of FP-biotin had been already described as a powerful tool for labeling and identifying not only serine hydrolases but any other proteins that interact with FPs (Liu et al. 1999). Labeling of serines and additional tyrosines on albumin has also been described *in vitro*, but with much lower reactivity (Ding et al. 2008). It has been argued that the possible role of OP binding by albumin binding OPs would be to reduce the amount of OP available for reaction with serine hydrolases (Peeples et al. 2005).

Albumin possesses several advantages as a biomarker of exposure, compared with the serine hydrolases. The OP-tyrosine bond is more stable and does not age like OPs adducted to active-site serines (Lockridge and Schopfer, 2010), allowing for a longer window of exposure detection and a more reliable identification of the OP involved in the exposure. Albumin is soluble in plasma and present in large quantities. In addition, oxime therapy does not displace the adducted OP from albumin (Read et al. 2010), allowing for identification of the OP of exposure after treating a patient. On the other hand, the rate of reaction of albumin with OPs is much slower compared with BChE (Li et al. 2008b). Thus, albumin is a less sensitive biomarker compared with BChE.

4.4. Carboxylesterase

Human carboxylesterase-1 (CES-1, EC 3.1.1.1, accession # 23141) is a trimeric 60-kDa serine esterase involved in OP detoxication or metabolic activation of various drugs and xenobiotics, as well as several environmental toxicants and carcinogens (Satoh and Hosokawa, 1998). It belongs to a large multigene family. CES-1 is primarily expressed in the endoplasmic reticulum and the cytosol of liver cells, but it is also found in lesser amounts in many other tissues, such as intestine, kidney, lung, gonads, monocytes and macrophages (Redinbo et al. 2003). CES catalyzes the hydrolysis of carboxylesters via a

two-step process in which the active-site serine is acetylated and subsequently deacetylated by the addition of water.

A half a century ago, Myers and Mendel (1949) reported that CES-1 (they called it aliesterase) was targeted by tri-*ortho*-cresyl phosphate (T*o*CP) and DFP. Later, they obtained results suggesting the preferential binding of some OPs to CES instead of ChEs, and hypothesized that one of the functions of CES could be to bind OPs so there would be less free OP to inhibit ChEs, thus modulating the negative effects of the OP exposure (Myers, 1952). The physiological evolution to protect against OP exposure is difficult to rationalize since synthetic OPs have been used in only very recent times. Following the hypothesis suggested by Myers, Su and co-workers administered 18 different OP insecticides to rats and studied the differential inhibition of liver, plasma and brain CES-1, compared with AChE. They observed that OPs have different potency as inhibitors of rat liver and plasma CES-1 (liver CES-1 being more sensitive to OPs than plasma CES-1), indicating that OP detoxication by CES-1 occurs predominantly in the liver, the site of OP bioactivation (Su et al. 1971). In addition, rat liver CES-1 was more susceptible to OP inhibition than liver, plasma and brain AChE, supporting the Myers' hypothesis. It should also be noted that CES-1 expression and activity can vary depending on the tissue and the organism analyzed (Imai, 2006). However, the ability of CES-1 to detoxify OPs depends on the quantity of enzyme (or the number of available binding sites in the enzyme) and the specific OP compound (Chanda et al. 1997). Since CES-1 is a stoichiometric scavenger (each CES-1 molecule can bind only one OP molecule), CES-1 is a high affinity/low capacity scavenger (Maxwell, 1992), in contrast to, for example, paraoxonase-1, a catalytic scavenger (Stevens et al. 2008).

Monocyte CES-1 is found in the outer cell membrane as an "ectoenzyme", where it can readily interact with the extracellular environment. It has an amino acid content similar to other esterases (Saboori and Newcombe, 1990). It has been demonstrated that OP compounds also inhibit human monocyte CES-1 activity (Emmett et al. 1985; Lee and Waters, 1977), providing another possible biomarker of exposure. FP-adducted murine plasma CES-1 has also been reported (Peeples et al. 2005).

One of the main drawbacks of monocyte CES-1 as a biomarker of OP exposure is the monocyte lifespan in circulation, which is only a few hours before they enter a tissue to differentiate into macrophages. On monocytes, CES-1 is much more sensitive to some OPs compared with AChE and BChE, and may be a useful biomarker if analyzed shortly after an exposure.

4.5. Neuropathy Target Esterase

NTE (EC 3.1.1.5, accession # Q8IY17) is a 150-kDa integral membrane protein anchored to the cytoplasmic face of the endoplasmic reticulum in both neurons and non-neural cells (Li et al. 2003). Despite its high capacity for hydrolyzing esters, it has no homology with any serine esterase or protease (Lush et al. 1998). NTE is primarily found in brain (especially in large neurons) with a substantially lower presence in spinal cord and sciatic nerve. It travels along axons by vesicular fast transport (Glynn et al. 1998). It is also found in non-neural tissues including intestine, spleen, liver, thymus, kidney, or testis as well as in lymphocytes (Dudek and Richardson, 1982; Glynn, 1999; Winrow et al. 2003).

In the 1930s, thousands of people in the United States were paralyzed by ingestion of adulterated alcoholic ginger extracts, contaminated with T*o*CPs. This was the first example of a large-scale OPIDN syndrome. In 1969, Martin Johnson reported the first evidence of *in vivo* phosphorylation of a specific esterase of the nervous system (NTE) by OPs that produced a delayed neurotoxic effect, later called OPIDN (Johnson, 1969). In his study with

hens, Johnson determined that NTE was resistant to some non-neuropathic OPs like paraoxon but sensitive to the neuropathic insecticide mipafox. Nonetheless, not all OPs that covalently inhibited NTE were neuropathic. The essential event initiating OPIDN by neuropathic OPs required the covalent phosphorylation of the NTE active-site serine, followed by generation of negatively charged species by hydrolysis of one of the phosphoryl ester bonds (Johnson, 1974). Johnson proposed that this charged group is responsible for the metabolic disturbances that lead to axonal degeneration.

Human NTE is very similar to a *Drosophila* neuronal protein called Swiss cheese (Sws), which is important in brain development and causes neurodegeneration when mutated (Lush et al. 1998). By analogy, this may be a physiological role of human NTE, with an involvement in neuronal-glial interaction during development, and in neurodegenerative diseases. In fact, NTE is expressed in neurons of mouse embryos from their earliest appearance in the developing nervous system (Moser et al. 2000); and the loss of NTE *in vivo* in a Cre/loxP mouse, with specific deletion of NTE in neuronal tissues, contributes to neurodegeneration characterized by vacuolization and neuronal loss (Akassoglou et al. 2004). Interestingly, the finding of an NTE-homologue in yeast suggests that NTE may have functions beyond the nervous system (Lush et al. 1998). Studies with the recombinant catalytic domain of NTE (known as NEST) indicated that lysolecithin (Quistad et al. 2003) was a physiological substrate of NTE. According to these findings, NTE is also a lysophospholipase with a possible contribution in phospholipid metabolism and membrane remodeling, the inhibition of which may lead to OPIDN (van Tienhoven et al. 2002).

The presence of NTE in blood lymphocytes (Dudek and Richardson, 1982), and the finding that its inhibition is correlated with NTE brain inhibition, poses NTE as another putative biomarker of exposure to neuropathic OP compounds. Lymphocyte NTE inhibition can be followed for up to 96 hours following OP exposure (Makhaeva et al. 2007).

4.6. Other biomarkers of exposure

The detection of OP adducts on the tyrosine-411 residue of albumin suggested that amino acid residues other than serines may also be modified by OP exposure (Means and Wu, 1979; Schopfer et al. 2005). In the late 1960s, binding of DFP to tyrosine residues of papain and bromelain was reported (Chaiken and Smith 1969; Murachi et al. 1965). Lockridge and co-workers reported OP insecticide and nerve agent binding to tyrosine residues of bovine alpha- and beta-tubulin (Grigoryan et al. 2008), and human and mouse transferrin (Li et al. 2009). *In vivo* detection of adducted tyrosines on brain tubulin from mice exposed to either chlorpyrifos or chlorpyrifos oxon has also been reported (Jiang et al. 2010). The authors suggest that adducts on tubulin could be responsible for neurotoxicity.

In addition to labeled tyrosines, adducted lysine residues have also been found in these and other proteins where tyrosines were also labeled by OPs, as observed with albumin (Lockridge and Schopfer 2010).

OP-adducted tyrosine and lysine residues in proteins with no active-site serine suggest that other proteins are modified by OPs at residues other than the active-site serines. These OP adducts may have biological implications in chronic exposures. Together with albumin, other tyrosine- and lysine-adducted proteins may well serve as biomarkers for OP exposure, although further studies are required to elucidate new proteins and sites of adduct formations that may serve as useful biomarkers of OP exposure.

5. Future perspectives

Biomonitoring of OP exposures requires better and more accurate analyses than the ChE activity measurements, especially since OPs have different affinities for biomarker proteins. The assays that are currently being used have several drawbacks and cannot provide information on the OP that generated the exposure. The Ellman assays need blood, the collection of which is considered invasive and sometimes logistically difficult. In addition to the post-exposure sample, a baseline sample is required in order to determine the percentage of inhibited activity. The detection of urinary metabolites does not require an invasive collection of the sample and can provide some information on the type of OP causing exposure. However, the half-life of these metabolites is really short. In addition, environmentally occurring OP-metabolites can also be detected in urine, causing a possible overestimation of the exposure.

The focus on MS analyses of OP-adducted biomarker peptides represents significant progress in the evaluation of OP exposures. MS has allowed development of new protocols for identifying and characterizing biomarkers of OP exposure. MS has become a powerful technique for discovering new biomarkers of exposure, specifically for the identification of post-translational modifications of proteins (e.g., OP adducts). Although highly sensitive and precise, MS approaches do have some drawbacks. The measurements can be difficult and time consuming (Costa, 1996). They also need to be carried out in a stable laboratory-based environment by well-trained technicians. The cost of MS instruments can be high. On the other hand, adduct measurement by MS can identify intact parent compounds or metabolites that are capable of forming covalent bonds with proteins, which represents a great advantage. A baseline sample is not required with MS analyses, eliminating the blood draw required for the baseline activity determination. In recent years, there have been significant improvements in the practical application of MS (Clarke and Hoofnagle, 2011). As a result, several clinical laboratories are beginning to use MS in routine assays, replacing traditional immunoassay instruments. These improvements include obtaining analytical data more rapidly with less expense, and an improvement in diagnostic accuracy. The implementation of MS as a routine technique needs additional improvement, but its future is promising.

There is much additional research that needs to be done in the area of biomarker characterization. New biomarkers that are being discovered need to be further investigated to ascertain whether or not they are better markers of exposure than the traditional ones. It is also desirable to find the best panel of biomarkers that should be monitored in case of exposure to an unknown OP. This is important since different OPs have different affinities for different biomarker proteins and some do not undergo the aging process providing more information about the OP associated with exposure. Overall, the identification and quantification of biomarkers of OP exposure using MS analyses will provide methods with increased accuracy and sensitivity. While modifications of biomarker proteins can provide information about the nature and extent of an OP exposure, the mechanism of toxicity may involve the modification of many other physiologically important proteins. Proteomic approaches under development will also provide avenues for exploring the physiological mechanisms of OP toxicity.

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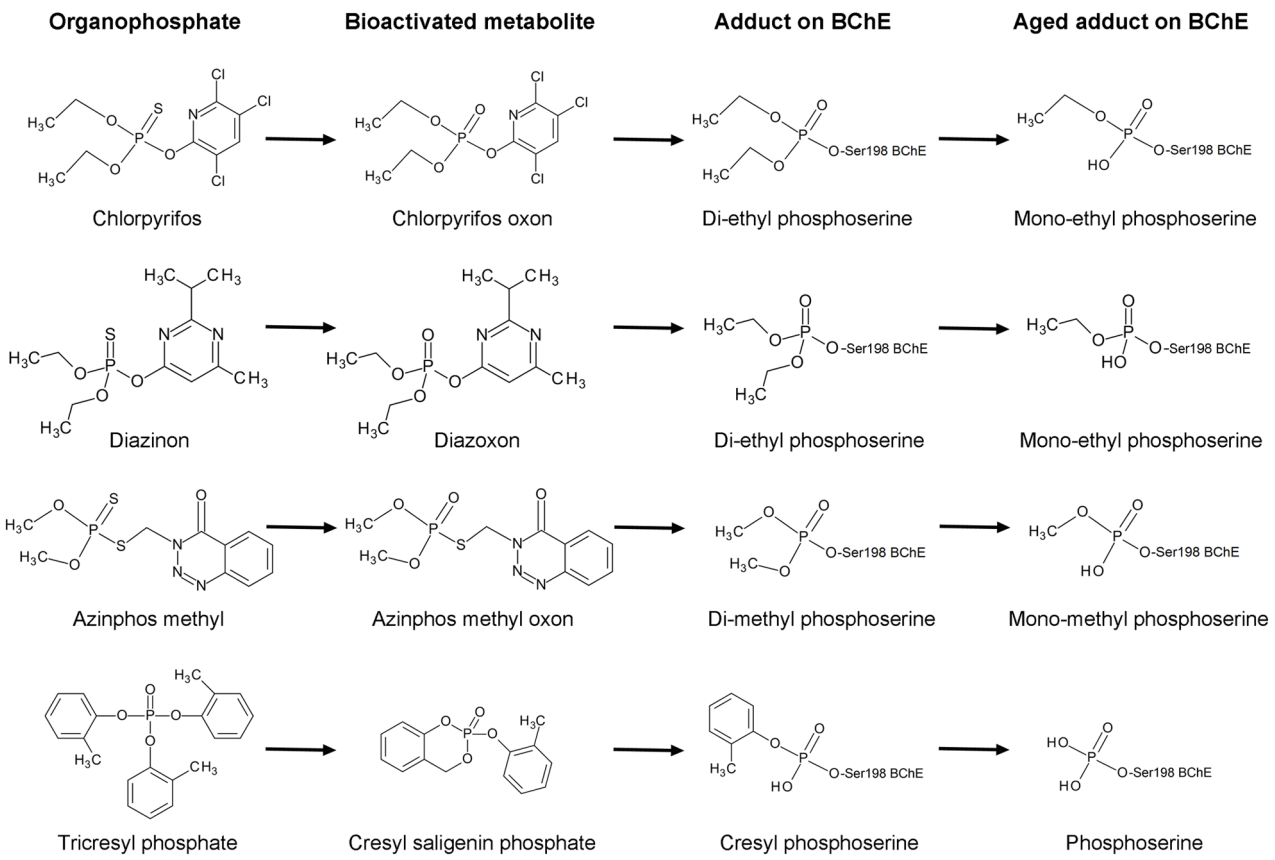

Figure 1.
Bioactivation, adduct formation and aging on BChE of the organophosphates chlorpyrifos, diazinon, azinphos methyl and tri-*ortho*-cresyl phosphate.

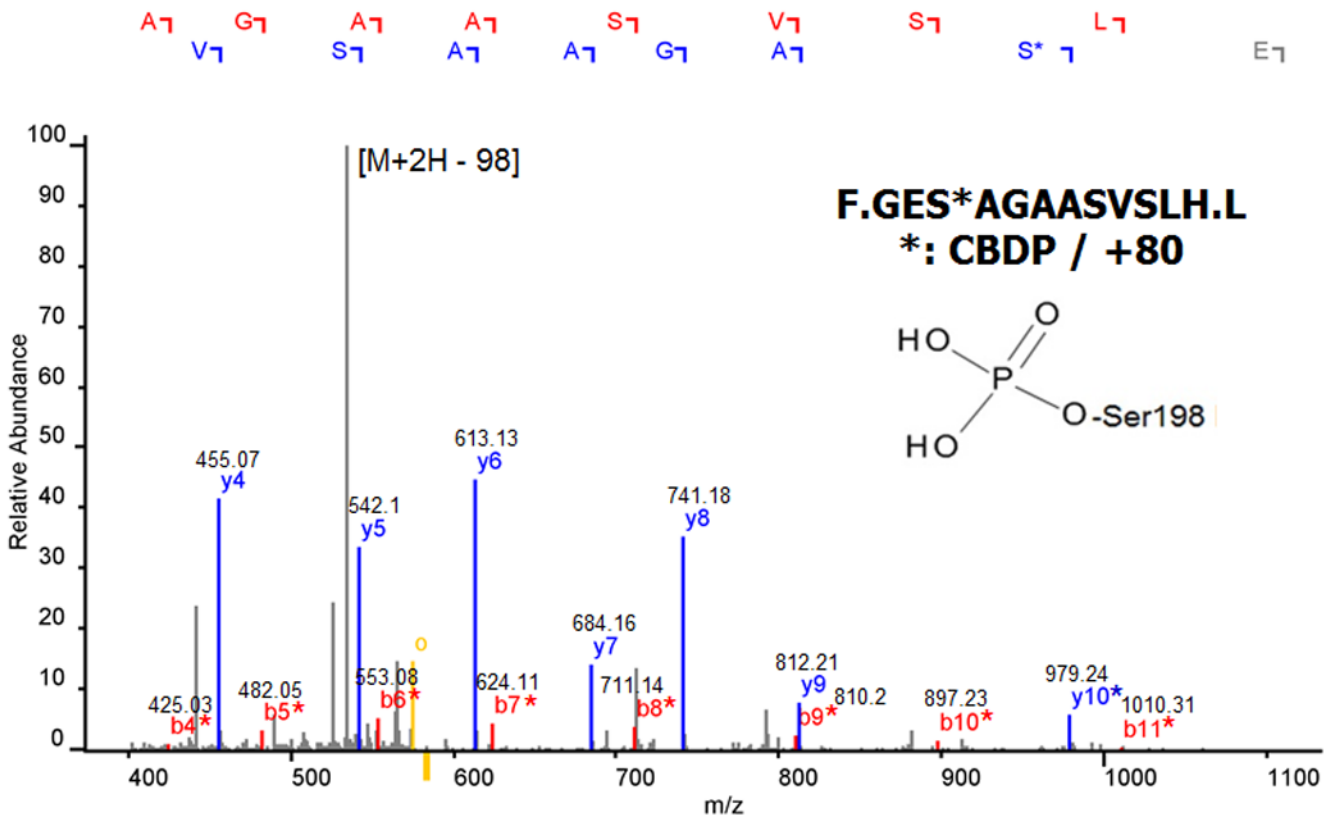

Figure 2.

Mass spectra showing the identification of BChE adducted active-site chymotryptic peptide by *in vitro* incubation with the active metabolite from tricresyl phosphate, cresyl saligenin phosphate (adapted from Marsillach et al. 2011). The asterisk (*) indicates an added mass of + 80 Da that corresponds to a phosphate group covalently bound to the serine 198 of the BChE active-site.