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Full Length Research Paper

HI-6 and obidoxime implication in oxidative stress, antioxidants level and apoptosis

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The oxime reactivators, obidoxime and asoxime (HI-6) are suitable for antidotal treatment after exposure to nerve agents. Although they are considered for use in humans under emergency situations, complete clinical studies are lacking as there were no clinical trials. We examined obidoxime and HI-6 in laboratory rats intramuscularly exposed to 25% of the median lethal dose (210 and 780 mg/kg) of each oxime reactivator and sacrificed 40 min after exposure. Brain and liver ferric reducing antioxidant power, reduced glutathione (GSH), glutathione reductase, thiobarbituric acid reactive substances, acetylcholinesterase, caspase 3 and glutathione S-transferase were assessed using standard protocols. We found significant depletion of liver and brain low molecular weight antioxidants. On the other hand, the depletion was partially recovered by an increase in the GSH level. Obidoxime was implicated in alteration of apoptotic processes in brain. Overall effects of oxime reactivators are discussed in this study. The pertinent adverse effects and strong modulation of disparate parameters of oxime reactivators, HI-6 and obidoxime are not well understood in antidotal treatment. We found strong impact of oxime reactivators on redox homeostasis and apoptotic processes.

Key words: Oxime, sarin, acetylcholinesterase, adverse effects, apoptosis, oxidative stress.

INTRODUCTION

Oxime reactivators are a group of antidotes suitable for causal treatment after exposure to organophosphorous pesticides and/or nerve agents (Bajgar, 2004). Nerve agents act as irreversible inhibitors of the enzyme, acetylcholinesterase (AChE; EC 3.1.1.7). Interaction of nerve agents with AChE results in alkylation of serine hydroxyl in the enzyme active site followed by inability to split the neurotransmitter, acetylcholine (Barthold and Schier, 2005). Oxime reactivators are able to break AChE - nerve agent complex providing active AChE and nerve agent moiety bound to the oxime reactivator (Ekstrom et al., 2009). The reactivation process is effective until spontaneous dealkylation of the organophosphate moiety, also called aging. The aging process is to the individual nerve agents (Sanson et al., 2009).

Despite the known molecular mechanism of AChE reactivation, the impact of oxime reactivators on the body functions during treatment processes is not well understood. Oxime reactivators can act as reversible inhibitors of AChE and antagonists at acetylcholine receptors (AChR) as reported, e.g., by Soukup et al. (2008). In earlier experiments, we found that the treatment process following exposure to nerve agents had adverse effects and modulated antioxidant barriers and oxidative stress (Pohanka et al., 2011a).

Our continuous effort is aimed at recognition of oxime reactivator implication in oxidative stress, apoptosis and other adverse effects. Two oxime reactivators (Figure 1), obidoxime and asoxime (HI-6), are prospective military purpose substances (Kassa, 2002). We chose these two reactivators for assessment of their impact on the cerebral cortex and liver in order to determine neurotoxicity and hepatotoxicity respectively. The doses of oxime reactivators were chosen as approximately ten times of recommended therapeutic dose (Kassa and Krejcova, 2003). This dose represents upper limit suitable for emergency application (Kassa, 2006; Pohanka et al., 2011a). The experiment was designed to assess

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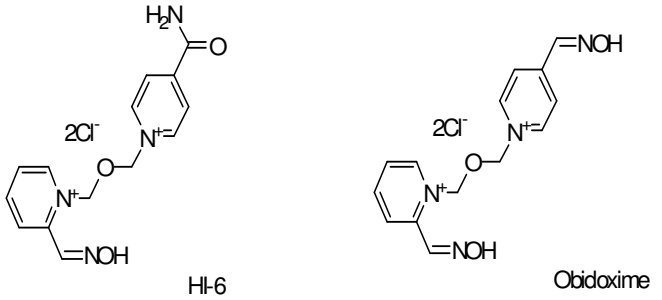

Figure 1. Structures of tested oximes.

oxidative stress in the time interval where the oxidative insult can be the most striking when considered in the recent paper (Pohanka et al., 2011a).

MATERIAL AND METHODS

Chemicals

Phosphate buffered saline (PBS) in tablets, caspase 3 colorimetric kit, 1-chloro-2,4-dinitrobezene, reduced glutathione and total protein kit TP100 were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Ethanol and sodium chloride were purchased from Penta (Prague, Czech Republic). Deionized water was prepared by MilliQ Ultrapure Water Purification system (Millipore, Billerica, Massachusetts, USA). All other chemicals were achieved from local sources in the analytical purity.

Animal exposures

Female Wistar rats (190 to 210 g body weight) were purchased from the Velaz Company (Prague, Czech Republic). The animals were kept under standard conditions (temperature 22±2°C, humidity 50±10% and light period 12 h day^{-1}). Feed and drinking water were provided *ad libitum*. The experiment was permitted and supervised by the Ethical Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic. In separate experiments, the toxicity of obidoxime and HI-6 were measured previously in our institution (Kassa and Cabal, 1999; Kassa, 2002). The median lethal dose (LD_{50}) for HI-6 and obidoxime, respectively, was calculated as 780 and 210 mg/kg.

The rats were divided into three groups, n = 6 animals. Controls were injected with 100 µl of saline only. The two experimental groups were intramuscularly (i.m.) injected with 25% of obidoxime or HI-6 (saline solution) LD_{50} in an amount 100 µl. After 40 min, the animals were euthanized using CO_2 narcosis.

Ex vivo assays

Two organs were sampled, that is, brain and liver. The frontal lobes of cerebral cortex and left lateral hepatic lobe were excised and then processed immediately at standard ambient temperature and pressure (SATP) conditions. 100 mg of tissue sample was immersed in 1 ml phosphate buffered saline (PBS) and mixed at 8,000 RPM using the Ultra-Turrax system (Ika, Werke, Staufen, Germany). The mixing lasted one minute and crude fragments were displaced by centrifugation at 1,000×*g* for five minutes.

AChE activity was assayed by the modified Ellman´s method in compliance with the reference (Pohanka et al., 2008). Caspase 3 (Casp3) activity was evaluated by a standard colorimetric kit as recommended by the producer. Assay was performed using a multichannel spectrophotometer and standard disposable 96-well microplates. Ferric reducing antioxidant power (FRAP) was carried out in order to estimate the total level of low molecular weight antioxidants. A standard protocol was used for this purpose. The experimental protocol was the same as in the reference (Pohanka et al., 2009). Glutathione-S-transferase (GST) activity was assayed using the following protocol in a slight modification of reference (Pohanka et al., 2011b): 10 µl 100 mM 1-chloro-2,4-dinitrobezene was poured with 10 µl of 100 mM reduced glutathione, 980 µl of PBS, and 50 µl of tissue homogenate. Absorbance was measured at one minute intervals. Enzyme activity was calculated considering the extinction coefficient 9,600 $M^{-1}cm^{-1}$. Reduced glutathione (GSH), glutathione reductase (GR) and thiobarbituric acid reactive substances (TBARS) were evaluated according to the cited protocol (Pohanka et al., 2011a). Total protein (TP) level was assayed by total protein kitin compliance with the protocol provided. The assessed markers were reached for the gram of protein. All *ex vivo* assays were carried out at SATP conditions.

Statistical analysis

Origin 8 SR2 (OriginLab Corporation, Northampton, MA, USA) was used throughout for experimental data processing, descriptive as well as inferential statistics. The significance of differences against controls was calculated using a one-way ANOVA with Scheffe´s test. Both probability levels of $p< 0.05$ and 0.01 were calculated for the examined parameters and groups of 6 specimens.

RESULTS AND DISCUSSION

The evaluated markers of oxidative stress (Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS), glutathione reductase (GR) and reduced glutathione (GSH)) are clearly depicted in Table 1. The levels of brain and liver GR and brain TBARS were not significantly altered. However, liver TBARS was extensively decreased. This could be due to slight metabolic depression as the TBARS marker can relate to the basal metabolism where residua coming from lipid peroxidation appear as a side product of liver oxidative metabolism (Lespine et al., 2001; Huang et al., 2008).

The level of low molecular weight antioxidants was markedly altered by oxime reactivators in terms of the FRAP value and the results are matched for obidoxime and HI-6. Liver is obviously more sensitive to the impact of oxime reactivators in terms of the FRAP value compared to brain. The total level of low molecular weight antioxidants was significantly ($P \leq 0.01$) decreased in the liver by oxime reactivators. HI-6 caused a molar decrease in low molecular weight antioxidants of 44%. Obidoxime was more striking as the decrease in antioxidants was 55%. Changes in the total level of low molecular weight antioxidants in the brain of exposed animals were not as extensive as in the case of liver. The FRAP value for cerebral cortex of HI-6 exposed animals was significantly ($P \leq 0.01$) decreased. The decrease was approximately

Table 1. Summarization of assessed oxidative stress, apoptosis and miscellaneous markers.

Marker	Organ	Control	HI-6	Obidoxime
FRAP (µmol/g)	Brain	3.96± 0.41	2.15±0.28 **	2.96±0.37
	Liver	9.73±1.32	5.47±0.43 **	4.45±0.22**
GSH (µmol/g)	Brain	0.268±0.033	0.567±0.054**	0.904±0.043**
	Liver	1.73±0.11	1.53±0.09	1.28±0.06*
GR (kat/ g)	Brain	34.3±2.2	30.0±2.3	30.7±1.4
	Liver	13.8±0.9	12.6±0.9	10.7±0.7
TBARS (µmol/g)	Brain	32.2±2.0	30.1±3.2	28.6±1.3
	Liver	47.2±5.1	28.3±3.1*	25.4±1.8 *
AChE (kat/g)	Brain	218± 13	218±17	188±11
	Liver	49.9±6.3	41.6±6.4	33.4±2.7
Casp3 (µkat/ g)	Brain	1.17±0.05	1.12±0.08	0.956±0.027*
	Liver	1.52±0.06	1.42±0.07	1.35±0.09
GST (kat/ g)	Brain	160±14	147±11	134±6
	Liver	92.1±5.9	80.6±5.6	70.0±3.7*

Value/gram of protein ± standard error of mean. FRAP, Ferric reducing antioxidant power; GSH, Reduced glutathione; GR, Glutathione Reductase; TBARS, thiobarbituric acid reactive substances; AChE, acetylcholinesterase; Casp3 - Caspase 3; GST - glutathione S-transferase; * = $p<0.05$, ** = $p<0.01$, n = 6 rats in each group.

45%; however, obidoxime caused no significant depletion of the total antioxidant capacity in the cerebral cortex. The depletion of total level of low molecular weight antioxidants was partially reversed by an increase in GSH. The cerebral cortex level of GSH was significantly ($P \leq 0.01$) increased after HI-6 as well as obidoxime. The GSH level in the cerebral cortex increased more than twice (HI-6) or four- times (obidoxime). Livers had relatively stable levels of GSH and only obidoxime caused partial depletion of GSH. The increase in relative as well as absolute levels of GSH in the cerebral cortex is probably a response to the depletion of another low molecular weight antioxidant. Though the GSH level is increased, the increase did not relate to the enzymes GR and GST. The experimental data are important for GSH that can cover depletion of the other antioxidants. The amelioration of GSH brain level and deprivation of the other antioxidant should be further investigated as it can present limitation in therapeutic applicability of oxime reactivators. The increased GSH in the brain can be beneficial after exposure to nerve agents as GSH is implicated in neuroprotection (Tsuru-Aoyagi et al., 2009; Wang et al., 2009; Dean et al., 2009). Pertinent exhaustion of GSH level in brain can lead to neurodegeneration (Aoyama et al., 2011). From this point of view, oxime reactivators could be plausibly implicated in neuroprotection of nerve agents exposed individuals by another way than AChE reactivation. On the other hand, the depletion of total level of low molecular weight antioxidants appoints at some adverse effects, caused by oxime reactivators application. Here, the effect of enhancing brain GSH level was recognized particularly for obidoxime. Moreover, the depletion of brain low molecular weight antioxidants was much lower for obidoxime than HI-6.The differences in HI-6 and obidoxime impact on brain can be caused by different penetration through blood brain barrier since blood brain barrier represents obstacle in oxime reactivators pharmacodynamics (Sakurada and Ohta, 2010; Wagner et al., 2010). Surprisingly, mice examined in our previous experiment were not so sensitive to 20% of HI-6 LD_{50} and alterations in low molecular weight antioxidants including GSH were of low grade for the whole brain and liver (Pohanka et al., 2011c).

Apart from the oxidative stress markers, we decided to investigate other markers to study the adverse effects of oxime reactivators. Activities of AChE, Casp3, GST and the level of proteins in the cerebral cortex and liver were investigated. The experimental data are shown in Table 1. Activitiy of AChE was not significantly influenced by oxime reactivators. Slight inhibition was caused by obidoxime; the second oxime, HI-6, had no effect on AChE activity despite its previously recognized inhibitory effect *in vitro* (Pohanka et al., 2011a). Reversible inhibitors of AChE are considered capable of initiating the AChE expression in a short time interval (Darreh-Shori

and Soininen, 2010). However, these effects should be elucidated more accurately. Slight inhibition can be covered by expression of new AChE.

The increased activity of Casp3 indicates apoptosis of cells. Casp3 may also be implicated in the regulation of neurogenesis apart from its main apoptotic function (D´Amelio et al., 2010). We proved alteration in Casp3 activity in the liver and cerebral cortex after obidoxime and HI-6 administration. Obidoxime seems to be more effective in Casp3 activity modulation when compared with HI-6. It caused significant ($0.01<P\leq0.05$) decrease of Casp3 activity in brain. Alteration in Casp3 activity can be hypothesized as a consequence of GSH production and alteration in level of the other low molecular weight antioxidants. This idea is supported by recent papers (Patnaik et al., 2010). For this reason, we infer that oxime reactivators do not participate in triggering of apoptosis. Hypothesis of GSH implication in Casp3 regulation seems to be plausible when the increase of GSH is considered after obidoxime and HI-6 application.

Differences between HI-6 and obidoxime can be explained by the effect of equitoxical dosage that does not correspond to the equimolar one. HI-6 is used in a higher dose than obidoxime as it is less toxic and the dose is equitoxical for the both oximes. Equitoxic dosage is widely recommended for oxime reactivators and use of less toxic reactivators is assumed to be more suitable as they can be applied in higher doses and more potent reactivation can be expected (Pohanka et al., 2010). The results presented here point toward limitations of treatment given the adverse effects found. The alteration in the followed markers after obidoxime and HI-6 application should be further investigated in order to understand adverse effects of the antidotal treatment. Understanding of adverse effects after oxime reactivators application can help to select optimal drugs for antidotal treatment. From this point of view, optimal antidotes should be not only highly be effective *in vitro*, but also low toxic and causing no or only minimal adverse effects (Sit et al., 2011; Kassa, 2003).

Conclusions

Oxime reactivators were found to induce adverse effects in brain and liver. We confirmed a significant effect of oxime reactivators in the modulation of the low molecular weight antioxidant level. We assessed effects caused by less toxic HI-6 and more toxic obidoxime. Obidoxime is implicated in apoptosis regulation. Surprisingly, HI-6 was not significantly influencing apoptosis despite four time higher applied dose than for obidoxime. Currently, the adverse effects of oxime reactivators are not well-understood and the data provide here are caveats in their clinical use.

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