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Impact of the Heavy Metals on the Activity of Xanthine Oxidoreductase

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Abstract

Background: Exogenous free radical production can occur as a result from the exposure to environmental pollutants, heavy metals (HM; Cd, Hg, Pb, Fe, and As), certain drugs (cyclosporine, tacrolimus, gentamycin, and bleomycin), chemical solvents, cooking (smoked meat, used oil, and fat), cigarette smoke, alcohol, and radiations [1]. HM (Cd, Hg, Pb, and As) and transition metals (Fe, Cu, Co, and Cr), acting as the powerful oxidative stress inducers, are responsible for various forms of nephropathy, as well as for some types of cancers [2]. In our current experiments we have investigated the impact of HM and pyridoxine on Xanthine Oxidoreductase (XOR; EC=1.17.3.2 - xanthine oxidase; EC= 1.17.1.4 - xanthine dehydrogenase) activity, which might serve as the source for the production of the reactive oxygen species. In our investigations we gave a privilege to the naturally occurred vitamin B6 in comparison to allopurinol based on knowledge regarding the negative side effects of this medicine.

Methods: The specific activity of the XOR in the brain of the rodents was measured by the measurement of the uric acid formation.

Results: In comparison with the control (0,5338±0,1542) both compounds allopurinol (0,21±0,1221) as well as pyridoxine (0,2616±0,1142) were suppressing the activity of XOR. Pyridoxine was diminishing the activity of XOR in the presence of the HM: for Cr^{6+} until the value of 0,1098±0,1098 in comparison with 2,3996±0,3541; for Pb^{2+} until 0,0130±7,9066e-3 vs 0,9492±0,3880; for Hg^{2+} until 0,6791±0,3572 vs 1,1604±0,6918 ($p<0,037489$ between the activation by Pb^{2+} and suppression under the impact of the pyridoxine; $p<0.05$ between the activation by Cr^{6+} and suppression under the impact of the pyridoxine).

Conclusion: We propose, pyridoxine might be used during acute HV intoxications.

Keywords: Allopurinol; Brain; Heavy Metals; Pyridoxine; Xanthine Oxidoreductase

Introduction

ROS (Reactive Oxygen Species) generation is the vitally important process. However, oxidative stress is determined as a misbalance between the formation of ROS and utilization. Generation of ROS in the normally functioning cells occurs in the organelles, as it was suggested previously, and transfers into the cytoplasm. Overwhelming amount of ROS might trigger the non-reversible cell death. It is supposed that mitochondria are the major reservoirs for ROS generation in most mammalian cells [3].

The respiratory chain is mainly localized in the inner membrane of the mitochondria and it is proved that the complexes I and III are the main responsible components of the chain for the production of the free radicals [4-7]. It is necessarily to mention, that 25% of free radicals formation occurs because of the protein folding in the ER (Endoplasmatic Reticulum) [8]. The leakage of Ca2+ ions into the cytoplasm also might triggers the production of ROS in mitochondria [9,10]. NADPH oxidases (NOXs) are the enzymes, functioning of which might be the key regulators in the pathological processes such as ischemia-reperfusion, diabetes, neurodegenerative diseases and atherosclerosis, as well as vessels related other diseases [11-14]. Hemoglobin and myoglobins might serve as a source for the formation of free radicals. Reduction of nitrite to ·NO under hypoxic conditions serves as a putative autoregulatory mechanism for capillaries and muscle [15]. Nitric oxide synthases are also might serve as a source of the ·NO [15]. The other enzyme, which will be highlighted in the frames of

this paper is Xanthine Oxidoreductase (XOR). Under normal circumstances, most amount of this enzyme exists in the form of NAD-dependent cytosolic dehydrogenase (XDH). However, in pathological conditions Xanthine Oxidase (XO) might be formed due to the limited proteolysis. XO as well as the XDH are two enzymes responsible for the last steps of purines metabolism, hydroxylation of a wide variety pyrimidine, pterin, and aldehyde substrates. The main reactions leading to the formation of the final products of the XOR functioning are the following:

- $\text{hypoxanthine} + H_2O + O_2 \leftrightarrow \text{xanthine} + H_2O_2$
- $\text{xanthine} + H_2O + O_2 \leftrightarrow \text{uric acid} + H_2O_2$
- $\text{superoxide ion } RH + H_2O + 2\ O_2 \leftrightarrow ROH + 2\ O_2^- + 2H^+$
- $\text{xanthine} + NAD^+ + H_2O \leftrightarrow \text{urate} + NADH + H^+$

The reactions are evidencing about the possible formation of the free radicals by the activity of XOR. From the other hand, exogenous free radical production can occur as a result from exposure to environmental pollutants, HV (Cd, Hg, Pb, Fe, and As), certain drugs (cyclosporine, tacrolimus, gentamycin, and bleomycin), chemical solvents, cooking (smoked meat, used oil, and fat), cigarette smoke, alcohol, and radiations [1]. Heavy (Cd, Hg, Pb, and As) and transition metals (Fe, Cu, Co, and Cr), acting as powerful oxidative stress inducers, are responsible for various forms of nephropathy, as well as for some types of cancers [2]. For instance, cadmium is a toxic metal with negative effects on health [16]. Occupational exposure is mainly from industrial processes. Smoking tobacco and contaminated food such as vegetables and rice are the main sources of general cadmium exposure. Blood cadmium (CdB) levels vary by region, age and ethnicity [16]. Previous studies have confirmed the pathogenic role of cadmium exposure in renal damage, bone destruction and cancer [17]. To determine whether CdB in Chinese adults is associated with serum uric acid and hyperuricemia, 2996 participants from the cross-sectional SPECT-China study were recruited. A positive relationship between serum uric acid and CdB was found in Chinese men after adjusting for the estimated glomerular filtration rate (eGFR), current smoking status, diabetes, dyslipidemia, hypertension and body mass index and in participants with eGFR > 60 mL/min per 1.73 m2. Further, the odds ratio of hyperuricemia increased with increasing CdB quartiles (P for trend < 0.05) in men. In conclusion, CdB was positively related to the serum uric acid level [18]. Investigations presented above are evidencing about the direct impact of the HV on the activity of the main enzyme responsible for the formation of uric acid and free radicals. Also, it is necessarily to mention, HM exposure is associated with cardiovascular diseases such as myocardial infarction (MI). Vascular dysfunction is related to both the causes and the consequences of MI. Combination of 1-month pre-exposure of $HgCl_2$ before MI changed the endothelial generation of oxidative stress induced by mercury exposure from NADPH oxidase pathway to XO (xanthine oxidase)-dependent ROS production [19]. It has been suggested that reactive oxygen intermediates (ROIs) may have a role in the genotoxic effects of lead (Pb^{2+}) and mercury (Hg^{2+}). By our investigations we have shown, inhibition of XOR is associated with the triggering of the regenerative processing after experimental stroke in rodents as well we in vitro [20-23].

It was shown, Pb^{2+} and Hg^{2+} (0.1–1 μM) had no effect on the activities of partially purified catalase, glutathione peroxidase, or glutathione reductase, important enzymes involved with antioxidant defense, but these metals stimulated the activities of copper - zinc superoxide dismutase (CuZn - SOD) and xanthine oxidase (XO).

Allopurinol (50 μM), a specific inhibitor of xanthine oxidase, inhibited the induction of H_2O_2 by Pb^{2+} (0.8–1 μM) and Hg_{2+} (1 μM) and also inhibited Pb^{2+} - and Hg^{2+} - induced mutagenesis. These results demonstrate that Pb^{2+} and Hg^{2+} disrupt the redox status of AS52 cells by enhancing the activities of CuZn - SOD and XO [24]. In our previous experiments, we have demonstrated that pyridoxine in the low concentrations (0.05 mg/ml) might prevent the activity of XOR [25]. In our current work, we have proposed that in rat brain HV, such as Cd^{2+}, Cr^{2+}, Pb^{2+}, Hg^{2+}, might trigger formation of ROS due to the induction of the XOR activity and pyridoxine in the low concentrations might inhibit that activity.

Material and methods

Justification of Animal Use

The experimental procedures with rodents were approved by the Ethical Committee of the H Buniatian Institute of Biochemistry of the National Academy of Science of Armenia (Reference Letter/ Protocol N 8. The Active International Registrations' Numbers of the Committee: IRB0001621, IORG 0009782). White laboratory rats were anesthetized by the injection of the pentobarbital in amount equal to the 10 mg/100 g of the weight.

Experimental Groups

The following experimental groups were analyzed: control (samples, which didn't contain any substrate and contained the enzymatic mixture), xanthine (samples, containing the substrate of XOR and enzymatic mixture), xanthine+pyridoxine (samples, containing the substrate of XOR and enzymatic mixture as well as pyridoxine), xanthine+allopurinol (samples, containing the substrate of XOR and enzymatic mixture as well as allopurinol), xanthine+iones of the heavy metals (IHM) (samples, containing the substrate of XOR and enzymatic mixture as well as IHM and xanthine+IHM+pyridoxine (samples, containing the substrate of XOR and enzymatic mixture as well as IHM and pyridoxine).

Homogenization of the Brain Tissue

All the reagents were purchased from Sigma-Aldrich. For 100 ml of the buffer it was used 0.87 g of the NaCl, 0.06 g of KH_2PO_4, 0.09 g Na_2HPO_4, 5 mM $MgCl_2$, 0.1.M Tris aminomethane, added 1 ml of Triton X 100, 200ug of tripsin inhibitors, as well as 0.001M $KNaC_4H_4O_6x4H_2O$. It was performed glass-glass homogenization during 20 minutes. The mixture was centrifuged at G= 8000 for 20 minutes. Supernatant was used for the experiments.

Xanthine Oxidase Activity Estimation by Determination of the Uric Acid Quantity in The Brain Tissue [26]

There were prepared 3 types of solutions, which were necessary for the final colorimetric detection of uric acid formation as the final product of XOR activity.

1. **Sodium Cyanide** - Urea Solution, containing 50 gm. of sodium cyanide, 300 gm. of urea, few gm. (5 or 6) of calcium oxide. For each 100 cc. of solution, it was added up to 1 gm. of crystallized disodic phosphate, $Na_2P0_4x5H_20$.
2. **Uric Acid Reagent** - It was used 100 gm. of sodium tungstate, dissolved in 200 ml of water. After all, it was added 20 ml of 85% phosphoric acid. H_2S was passed through the solution for 20 minutes, and during this process (at the end of 3 to 4 minutes) it was added another 10 ml. of 85% of phosphoric acid. After all, it was added 300 ml. (1.5 volumes) of alcohol. The mixture separated into two layers. The lower layer was immediately transferred into a previously weighed 500 ml. flask; the upper layer was discarded. Further we added water to the mixture in the weighed flask until the volume of the contents reached 300 ml. The solution was boiled a few minutes to remove the H_2S. After all, the flame was removed; it was added 20 ml of 85 % phosphoric acid. The flame was removed, and decolorized with a few drops of bromine.

Lithium carbonate (12 gm) was transferred to a 500 ml beaker, added first 25 ml of phosphoric acid, and then slowly 150 ml of water. The solution was boiled to remove the CO_2. The lithium phosphate solution was cooled, mixed with the concentrated uric acid reagent, and diluted to 1 liter.

3. **Standard Uric Acid Solution** - It was added 0.6 gm. of lithium carbonate to 150 cc. of water. The solution was heated. After cooling, it was added 1 gm of the uric acid into the lithium carbonate solution. It was added 20 cc. of 40 % formalin. Also, it was added a few drops of methyl orange solution and finally added 25 cc. of normal sulfuric acid. XOR activity was measured by the addition of xanthine and/or allopurinol (0.4 ug per 1 ml) into the final mixture, which was incubated with the biological solution for one hour at 37^0C. The amount of the added HM was equal to Pb^{2+} 40 mg, Cd^{2+} 77 mg, Cr^{6+} 4.9 mg, Hg^{2+} 3.6 mg per 1 ml of the mixture. Pyridoxine was added into the solution in 0.05 mg amount. NAD+ was added in the amount equal to 0.01 mg. It was used Cary 60 spectrophotometer (Agilent, USA). The absorption of "blue complex" generated with uric acid after addition of the above presented solutions was measured at 660 nm of the wavelength. The number of the experiments was varying from 4-6. Protein determination was performed by Bradford method.

Statistics

In our calculations we have used t-test (student) for pair comparison as well as ONE-WAY-ANOVA for the calculation of the significance of the comparable all groups. The results were considered statistically significant when p was lower or equal to 0.05. In some calculations we used t-student test. It was used Sigma Stat 10 for the calculation of statistics. Sigma Plot 10 was used for the building of the graphs.

Results

The Impact of the Allopurinol and Pyridoxine On the Activity of XOR

In comparison with the control (0,2089±0,1529) in the presence of the substrate the activity of the enzyme was elevated until 0,5338±0,1542. In the presence of the pyridoxine that activity was decreased until 0,2616±0,1142, whereas allopurinol diminished the activity of the enzyme until 0,2100±0,1221 (Figure 1).

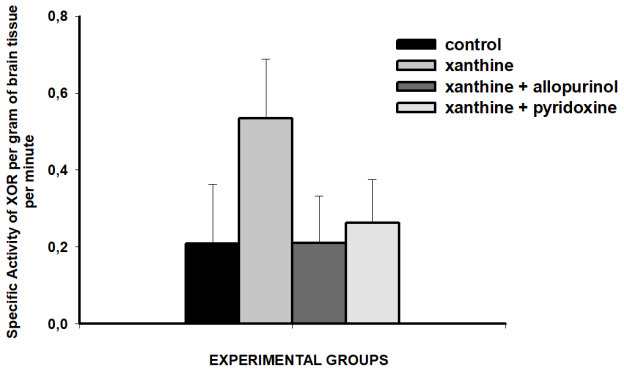

Figure 1: The impact of the allopurinol and pyridoxine on the activity of XOR. The number of the experiments was equal to 4.

The Influence of HM On the Activity of the XOR

In accordance to our investigations the HM have the activity elevating impact on XOR. Cd^{2+} elevated the activity of the XOR in comparison with the control 0,5338±0,1542 vs 0,6488±0,3635. Cr^{6+} ions elevated the activity of XOR until 2,3996±0,3541 ((Figure 2); $p^{\#} < 0.05$), Pb^{2+} - until 0,9492±0,3880 and Hg^{2+} - until 1,1604±0,6918.

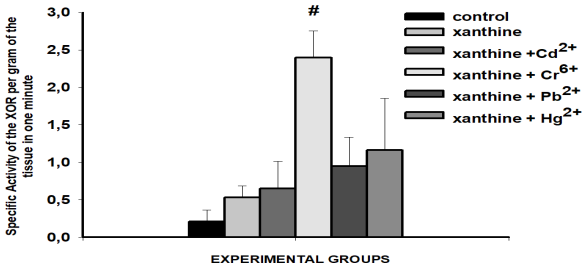

Figure 2: The influence of HM on the activity of the XOR.

The number of the samples were varying in the groups from 4-5. There were used ONE-WAY-ANOVA as well as student t-test for calculation of the statistics. The difference between Cr^{6+} ions containing group and the group containing enzymatic mixture and xanthine was statistically significant and $p^{\#}<0.05$ (t-test).

Suppression of XOR activity in the presence of HM and pyridoxine

Pyridoxine was able mostly to diminish the activity of the XOR in the presence of the HM. The impact of the ions of the Cd^{2+} were switching the value until 0,8522±0,3461, for Cr^{6+} until 0,1098±0,1098; for Pb^{2+}-0,0130±7,9066e-3 and for Hg^{2+}-0,6791±0,3572 (p#<0,037489 between the activation of Pb^{2+} and that suppression under the impact of the pyridoxine; p#<0.05 between the activation of Cr^{6+} and that suppression under the impact of the pyridoxine) in comparison with the controls, where there were added the substrate and HM (Figure 3).

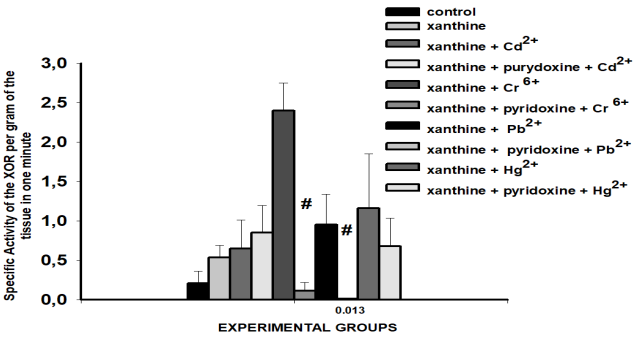

Figure 3: Suppression of XOR activity in the presence of HM and pyridoxine.

The number of the samples were varying in the groups from 4-6. There was used ONE-WAY-ANOVA as well as student t-test for calculation of the statistics. The difference between Cr^{6+} ions containing group vs the group containing enzymatic mixture +xanthine+ Cr^{6+}+pyridoxine was statistically significant and $p^{\#}<0.05$ (t-test). Also, the difference between Pb^{2+} ions containing group vs the group containing enzymatic mixture +xanthine+ Pb^{2+}+pyridoxine was statistically significant and $p^{\#}<0.05$ (t-test).

Discussion

Allopurinol is the known inhibitor of XOR. However, in our experiments we have made the most vivid emphasize on the inhibition of XOR by pyridoxine. First, because the pyridoxine is the naturally occurred compound and don't have side effects during the utility in the low doses whereas the allopurinol is known by its none or difficult tolerated nature due to the triggering the over activity of immune system. Negative side effects of allopurinol include hypersensitivity syndrome, which manifests as rash, eosinophilia, leukocytosis, fever, hepatitis and progressive kidney failure, with high mortality rates [27]. Previously, we have demonstrated that XOR activity in the neuronal cell culture obtained from the human embryo brain cells [28] was diminished in the presence of the pyridoxine. Further, we have purified the XOR and determined concentration dependence influence of pyridoxine on the activity of purified XOR. We have concluded that in the presence of the low concentrations of pyridoxine XOR activity was suppressed whereas the high concentration had the opposite effect [25]. We propose, that previously as well as now the interaction of XOR and the pyridoxine is explained by the primer stereo interaction of the effector and enzyme. We propose, that pyridoxine might be used during acute HM intoxications.

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