Acta Biologica Hungarica 58 (4), pp. 359–367 (2007)
DOI: 10.1556/ABiol.58.2007.4.3

IN VITRO EFFECTS OF ALLOXAN/COPPER COMBINATIONS ON LIPID PEROXIDATION, PROTEIN OXIDATION AND ANTIOXIDANT ENZYMES

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(Received: September 5, 2006; accepted: December 15, 2006)

The *in vitro* effects of alloxan and the product of its reduction dialuric acid (alone or in combination with copper ions) on lipid peroxidation, carbonyl content, GSH level and antioxidant enzyme activities in rat liver and kidney have been studied. The effects of Cu^{2+}/alloxan and Cu^{2+}/dialuric acid were compared with those of Fe^{3+}/alloxan and Fe^{3+}/dialuric acid. Unlike alloxan, dialuric acid increased liver and kidney lipid peroxidation; similar effects were registered in the presence of Fe^{3+}. In the presence of Cu^{2+}/dialuric acid, the lipid peroxidation was strongly inhibited and vice versa – the liver protein oxidation was increased. Alloxan and dialuric acid, as well as their combinations with Fe^{3+} had no effect on the total GSH level. Both substances did not affect the Cu^{2+}-induced changes in GSH level, glucose-6-phosphate dehydrogenase and gluthatione reductase activities. In contrast, Cu^{2+} had no effect on dialuric-acid induced changes in gluthatione peroxidase and superoxide dismutase activities. The present *in vitro* results, concerning the metal dependence of the effects of alloxan and dialuric acid, are a premise for *in vivo* study of alloxan effects in metal-loaded animals.

Keywords: Alloxan – dialuric acid – copper – lipid peroxidation – antioxidant enzymes

INTRODUCTION

The cytotoxic action of alloxan (***A***), used for inducing of experimental diabetes, is connected with the participation of reactive oxygen species (ROS); ***A*** is reduced to dialuric acid ($\boldsymbol{AH_2}$), the oxidation of the latter leading to production of alloxan radical ($\boldsymbol{AH}\cdot$), superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) [7, 13, 20]. In the presence of transition metals (mainly iron), a production of hydroxyl radicals (·OH) is also registered [24; this cell-damaging radical is believed to be the main toxic agent in ***A***-induced diabetes, 7, 8, 16, 20, 32].

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Using the deoxyribose test [12], we found that the $\boldsymbol{AH_2}$-dependent deoxyribose degradation was changed in a different manner by the presence of metal ions (Fe^{3+}, Cu^{2+}, V^{5+} or V^{4+}), H_2O_2, metal reducers or metal chelators [1, 17]. The copper effects on ***A***- and $\boldsymbol{AH_2}$-induced inhibition of O_2^{-}-provoked nitro blue tetrazolium (NBT) reduction [2] and the vanadium effects on $\boldsymbol{AH_2}$-induced lipid peroxidation in rat liver and kidney [3] were also found to differ from those of iron. In addition, an insulin-mimetic activity of some metals (vanadium, lithium, selenium, molybdenum) has been reported [5, 21, 22, 25, 28, 29, 31]. All these findings justify our investigations on the role of the different transition metals in the ***A***-action.

The aim of this study was to investigate the *in vitro* effects of alloxan and its reduction product – dialuric acid, on lipid peroxidation, protein oxidation and antioxidant enzyme activities in rat liver and kidney preparations in the presence of copper. The results were compared with those, observed in the presence of iron.

MATERIALS AND METHODS

Chemicals

Alloxan was purchased from BDH; dialuric acid – from Riedel-de Haen AG; NBT – from Serva; H_2O_2 and riboflavin – from Merck. All other reagents were analytical grade; all solutions were prepared with de-ionized water.

Animals

Male Wistar rats, weighing 180–200 g, were given free access to tap water and a standard lab diet. The animals were starved 24 h and then killed under light ether anaesthesia.

Tissue preparations

Liver, perfused with chilled 0.15 M KCl, and kidney were homogenized in cold 0.15 M KCl –10 mM potassium phosphate buffer, pH 7.4. After centrifugation of the 10% liver and kidney homogenates at 600 *g* for 10 min, "post-nuclear" preparations were obtained. Part of these homogenates and the supernatants, obtained after centrifugation of homogenates at 12,000 *g* for 20 min ("post-mitochondrial" supernatant), were used in the present experiments; samples (2 mg protein/ml buffer) were incubated for 15 min at 0 °C in the presence and in the absence of adding alloxan, dialuric acid, $FeCl_3$ or $CuCl_2$.

Analytical methods

Protein content was measured by the method of Lowry et al. [19].

Protein carbonyl content (in "post-nuclear" homogenates) was measured at 366 nm, according to Reznick and Parker [27].

Lipid peroxidation (LP) was determined by the amount of thiobarbituric acid reactive substances (TBARs), formed in fresh "post-nuclear" homogenates (mg protein/ml) after 60-min incubation at 37 °C and expressed in nmoles malondialdehyde (MDA)/mg protein [15]. The 600 nm absorbance was considered to be a non-specific baseline and was subtracted from A_{532}.

Glutathione (GSH) level in "post-nuclear" homogenates was measured at 412 nm by the method of Tietze [33].

Glutathione peroxidase (GSH-PER) activity in "post-mitochondrial" supernatant (with t-butylhydroperoxide as a substrate) was measured by the method of Gunzler et al. [9].

Glutathione reductase (GSSG-RED) activity in "post-mitochondrial" supernatant was measured by the method of Pinto and Bartley [26].

Glucose-6-phosphate dehydrogenase (Glu-6-P-DH) activity in "post-mitochondrial" supernatant was determined after Cartier et al. [6].

Superoxide dismutase (SOD) activity in "post-mitochondrial" supernatant was determined according to Beauchamp and Fridovich [4]. The samples were lighted for 5 min with a 250-W HgL lamp and the inhibition of O_2^--provoked nitro blue tetrazolium (NBT) reduction was measured at 560 nm; one unit of SOD activity is the amount of the enzyme, inhibiting the NBT reduction by 50%.

Statistical analysis

The results were statistically processed by Student *t*-test; $P < 0.05$ being accepted as the minimum level of statistical significance of the established differences. The number of rats used during the experiments is given under the tables and figure.

The experiments have been performed according to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), and the rules of the Ethics Committee of the Institute of Physiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Services).

RESULTS

$\boldsymbol{AH_2}$, the reduction product of ***A***, increased the amount of TBARs (an index of lipid peroxidation) formed in rat liver and kidney homogenates (Table 1); the ***A***-effect was similar to that of $\boldsymbol{AH_2}$, but only in the presence of GSH (a metal and alloxan reducer). Both AH_2^- and A/GSH-induced LP was intensified after Fe^{3+}-, but not after Cu^{2+}-addition to the reaction mixture. Unlike Fe^{3+} (a good LP-inductor), Cu^{2+} (in the

Table 1
Effects of alloxan and dialuric acid on lipid peroxidation

Additions	Controls	100 μM Fe^{3+}	100 μM Cu^{2+}
	Liver lipid peroxidation		
Controls	1.8 ± 0.09	5.0 ± 0.14*	0.3 ± 0.03*
+100 μM alloxan	2.0 ± 0.14	5.6 ± 0.19*	0.3 ± 0.02*
+100 μM dialuric acid	3.4 ± 0.10*	7.9 ± 0.16**	0.3 ± 0.03*
+100 μM alloxan/100 μM GSH	3.2 ± 0.07*	7.4 ± 0.37**	0.4 ± 0.03*
+100 μM GSH	1.0 ± 0.08*	5.2 ± 0.23*	0.3 ± 0.04*
	Kidney lipid peroxidation		
Controls	1.1 ± 0.12	2.1 ± 0.17*	0.3 ± 0.03*
+100 μM alloxan	1.4 ± 0.11	2.2 ± 0.22*	0.3 ± 0.02*
+100 μM dialuric acid	2.4 ± 0.11*	3.6 ± 0.16**	0.4 ± 0.07*
+100 μM alloxan/100 μM GSH	1.7 ± 0.04*	4.8 ± 0.08**	0.3 ± 0.03*
+100 μM GSH	1.3 ± 0.14	3.6 ± 0.15**	0.3 ± 0.03*

The values represent the mean ± SEM of 7 animals and are expressed in nmoles MDA/mg protein, using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$. Statistically significant differences versus corresponding controls (*in the absence of metal ions and **in the presence of metal ions) at $P < 0.05$.

concentration used) strongly decreased the TBARs formation in both tissue preparations, $\boldsymbol{AH_2}$ and ***A***/GSH failing to change it.

$\boldsymbol{AH_2}$ increased the carbonyl content (an index of protein oxidation) in liver, but the effect of $\boldsymbol{AH_2}/Cu^{2+}$ was stronger (Fig. 1). The $Cu^{2+}/\boldsymbol{AH_2}$ effect on protein oxidation

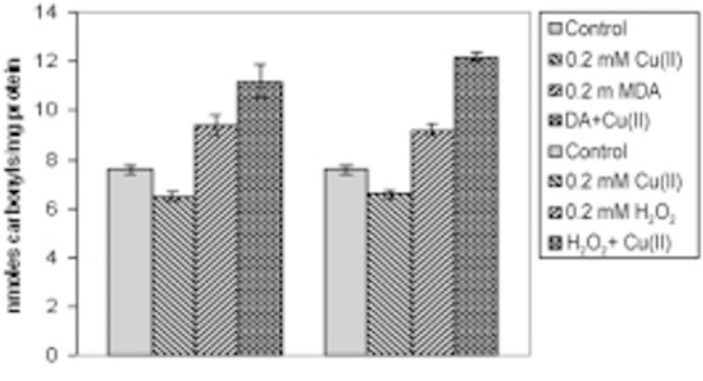

Fig. 1. Effects of dialuric acid on carbonyl content in rat liver. The values represent the mean ± SEM of 5 animals and are expressed in nmoles carbonyls/mg protein, using a molar extinction coefficient of $2.2 \times 10^4\ M^{-1}cm^{-1}$. *Statistically significant differences versus controls at $P < 0.05$

Table 2
Effects of alloxan and dialuric acid on GSH level in rat tissues

Additions	Controls	100 μM Fe^{3+}	100 μM Cu^{2+}
	Liver GSH level		
Controls	3131 ± 195	2721 ± 220	2434 ± 104*
+100 μM alloxan	2836 ± 119	2843 ± 292	2688 ± 164*
+100 μM dialuric acid	2864 ± 225	2787 ± 221	2463 ± 226*
	Kidney GSH level		
Controls	1139 ± 65	1338 ± 149	897 ± 54*
+100 μM alloxan	1201 ± 101	1191 ± 131	854 ± 91*
+100 μM dialuric acid	1066 ± 42	979 ± 114	871 ± 62*

The values represent the mean ± SEM of 7 animals and are expressed in ng GSH/mg protein. * Statistically significant differences versus controls (in the absence of metal ions) at $P < 0.05$.

Table 3
Effects of alloxan and dialuric acid on antioxidant enzyme activities in rat liver

Additions	Controls	100 μM Fe^{3+}	100 μM Cu^{2+}
	GSH-PER activity		
Controls	218 ± 13.4	219 ± 11.3	207 ± 17.1
+100 μM alloxan	5*	9*	9*
+100 μM dialuric acid	4*	7*	7*
	GSSG-RED activity		
Controls	36 ± 2.5	39 ± 4.6	28 ± 1.9*
+100 μM alloxan	33 ± 4.2	38 ± 4.2	29 ± 1.8*
+100 μM dialuric acid	43 ± 3.2	44 ± 5.7	18 ± 1.4**
	Glu-6-P-DH activity		
Controls	29 ± 2.9	29 ± 2.5	14 ± 2.2*
+100 μM alloxan	33 ± 4.2	28 ± 3.4	15 ± 1.6*
+100 μM dialuric acid	38 ± 4.9	34 ± 4.0	13 ± 1.2*
	SOD activity		
Controls	74 ± 5.6	80 ± 3.7	92 ± 6.8
+100 μM alloxan	155 ± 5.5*	144 ± 9.2*	136 ± 11.9*
+100 μM dialuric acid	131 ± 15.9*	133 ± 12.1*	132 ± 11.5*

The values represent the mean ± SEM of 7 animals. GSH-PER, GSSG-RED and Glu-6-P-DH activities are expressed in in nmoles NADP(H)/min/mg protein, using a molar extinction coefficient of 6.22×10^6 M^{-1} cm^{-1} and SOD is expressed in U/mg protein. Statistically significant differences versus corresponding controls (* in the absence of metal ions and ** in the presence of metal ions) at $P < 0.05$.

Table 4
Effects of alloxan and dialuric acid on O_2^--provoked NBT reduction

Additions	Controls	100 μM Fe^{3+}	0.05 μM Cu^{2+}	100 μM Cu^{2+}
	0	3±2.3	12±1.3*	92±1.1*
+10 μM Alloxan	24±2.1*	–	–	–
+50 μM Alloxan	62±1.9*	61±5.2*	44±1.5**	96±0.3*
+100 μM Alloxan	76±2.7*	70±4.9*	57±2.4**	97±0.6*
+10 μM Dialuric acid	21±2.3*	–	–	–
+50 μM Dialuric acid	53±1.5*	60±3.9*	38±1.3**	96±0.4*
+100 μM Dialuric acid	67±1.6*	72±2.2*	45±0.7**	96±0.5*

The values represent the mean±SEM of 10 experiments and are expressed in % inhibition of NBT reduction. Statistically significant differences versus corresponding controls (* in the absence of metal ions and ** in the presence of metal ions) at $P < 0.05$.

was similar to that of Cu^{2+}/H_2O_2; it might be mentioned that the latter combination presents Fenton-like system, generating ·OH radicals. The effects of ***A*** and $\boldsymbol{AH_2}$ on GSH-level and the antioxidant enzyme activities in liver and kidney preparations in the absence and in the presence of Fe^{3+} and Cu^{2+} were also measured. ***A*** and $\boldsymbol{AH_2}$, alone or in combinations with Fe^{3+}, had no effect on the total GSH level; besides, the both substances did not change the Cu^{2+}-induced decrease of GSH level (Table 2). Unlike Fe^{3+}, Cu^{2+} inhibited Glu-6-P-DH and GSSG-RED activity but this Cu^{2+}-induced inhibition was not affected by ***A*** and $\boldsymbol{AH_2}$; only an additional decrease of Glu-6-P-DH activity in the presence of $\boldsymbol{AH_2}$ was observed (Table 3). Conversely, Cu^{2+} had no effect on ***A***- and $\boldsymbol{AH_2}$-induced decrease of GSH-PER activity and $\boldsymbol{AH_2}$-induced increase of SOD activity.

As seen in Table 4, ***A*** and $\boldsymbol{AH_2}$ dose-dependently inhibited O_2^--provoked NBT-reduction. Administered alone, 100 μM Fe^{3+} did not change NBT reduction both in the absence and in the presence of ***A*** and $\boldsymbol{AH_2}$. In a concentration of 100 μM, Cu^{2+} led to full inhibition of NBT reduction and on this background we failed to register any additional effects of ***A*** and $\boldsymbol{AH_2}$; however, a tendency to a decrease in ***A***- and $\boldsymbol{AH_2}$-induced inhibition of NBT reduction was observed in the presence of a low Cu^{2+} concentration (0.05 μM).

DISCUSSION

It is well known that ·OH radicals are highly reactive and damaging for the cell and that their formation is possible only in the presence of transition metals and H_2O_2 [11].

When transition metal ions and $\boldsymbol{AH_2}$ were present together in the reaction mixture, the following reactions might be taken in consideration [14, 24]:

$$\boldsymbol{AH}_2 + O_2 \rightarrow O_2^- + \boldsymbol{AH}\cdot + H^+$$
$$\boldsymbol{AH}_2 + O_2^- + H^+ \rightarrow \boldsymbol{AH}\cdot + H_2O_2$$
$$\boldsymbol{AH}\cdot + O_2^- + H^+ \rightarrow \boldsymbol{A} + H_2O_2$$
$$\boldsymbol{AH}_2 + Me^{n+1} \rightarrow \boldsymbol{AH}\cdot + Me^n + H^+$$
$$\boldsymbol{AH}\cdot + Me^{n+1} \rightarrow \boldsymbol{A}\cdot + Me^n + H^+$$
$$Me^n + H_2O_2 \rightarrow Me^{n+1} + OH^- + \cdot OH$$

In O_2-presence, the formed ·OH radicals lead to peroxidation of numerous organic molecules, including proteins [10, 11]; these radicals attack the side chains of amino acid residues at the metal binding site, leading to protein modification and increase in carbonyl content [30]. Hence, the increase of $\boldsymbol{AH}_2$-evoked carbonyl content in Cu^{2+} presence (Fig. 1) might be explained with ·OH radicals formation. The identity in the effects of $Cu^{2+}/\boldsymbol{AH}_2$ and Cu^{2+}/H_2O_2 (Fenton-like system, generating ·OH radicals) on liver carbonyl content is an additional confirmation for ·OH-formation.

It is known that copper ions display high affinity for both NH_2- and SH-groups, occurring in proteins. Specialized proteins containing clusters of these groups transport and store copper ions, hampering their potential toxicity; this mechanism may be overwhelmed under copper-overload conditions, in which copper ions bind to the SH-groups of proteins, non-related to copper metabolism. Thus, indiscriminate copper binding may damage protein structure, modifying its biological functions [18]. It might be suggested that the protein damage induced by copper in μM concentrations is a reason for the decreased activity of GSH transferase [18], GSH reductase and Glucose-6-P-DH (Table 3). In addition, the results presented in Table 4 suggested that the increase in tissue SOD activity in the presence of ***A*** and $\boldsymbol{AH}_2$ (Table 3) is putative (SOD-like activity). These findings must be taken into consideration, when discussing the *in vitro* effects of other redox-cycling substances, like $\boldsymbol{A}/\boldsymbol{AH}_2$, on tissue SOD activity.

Probably, the strong inhibition of liver and kidney lipid peroxidation in the presence of Cu^{2+} or $Cu^{2+}/\boldsymbol{AH}_2$ (Table 1) is due to the high Cu^{2+}-concentration (100 μM) used in the present study. This is in accordance with data of Letelier et al. [18] that Cu^{2+} in nM concentrations range induces a significant lipoperoxidation, while it is non-detectable at increasing Cu^{2+}-concentrations to ≥50 μM. Unlike $Cu^{2+}/\boldsymbol{AH}_2$, the combination of $\boldsymbol{AH}_2$ (O_2^--generating system) with Fe^{3+} led to a higher formation of TBARs in liver and kidney than $\boldsymbol{AH}_2$ and Fe^{3+} alone (Table 1). This is in accordance with the results obtained in the system xanthine oxidase + ADP/Fe^{3+}, where the O_2^- and H_2O_2, produced from xanthine oxidase support iron-catalyzed lipid peroxidation through their participation in redox reactions of iron [23].

In conclusion, the present *in vitro* results appeared to be a premise for studying and comparing the *in vivo* effects of alloxan in animals, pretreated with copper or iron. Thus, further *in vitro* and *in vivo* studies could be useful for understanding the role of these metal ions in ***A***-induced diabetes.

ACKNOWLEDGEMENT

This study was supported by Grant MU-B-1001 from the National Research Fund, Bulgaria.

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