The Role of Cadmium in Proteins Glycation by Glucose: Formation of Methylglyoxal and Hydrogen Peroxide in Vitro

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***Abstract*—Cadmium (Cd) is a heavy metal that be a source of concern for industrial workers and it was proposed in the formation of advance glycation end products (AGEs) such as methylglyoxal (MG). MG have recently attracted much attention because of their possible clinical significance in chronic and age-related diseases. Based on previous research, methylglyoxal formation can be accelerated by metals in vitro. The role of Cd in the formation of MG and hydrogen peroxide has not been much studied. Thus, our study aims to measure the formation rate of MG and hydrogen peroxide in the presence of Cd in vitro. This research was divided into 4 groups (1 control group and 3 treatment groups), than we set carbonyl compound assay, methylglyoxals assay, and hydrogen peroxide assay. For analyzing of the data, SPSS software version 17 was used and was examined by ANOVA and regression correlation test. For all outcomes, a nominal p-value of $p < 0,05$ was considered significant. We found that there are significant correlation between Cd exposure on the formation of hydrogen peroxide and methylglyoxal ($p < 0,05$) in nonenzymatic glycation of proteins by glucose. The increased Cd level accelerate the formation of methylglyoxal and hydrogen peroxide.**

***Index Terms*—Cadmium, kinetics first order, methylglyoxal, glycated protein, hydrogen peroxide**

I. Introduction

Cadmium (Cd) is typically a heavy metal used in rechargeable batteries and for the production of special alloys. Although emissions in the environment have markedly declined in most industrialized countries, Cd remains a source of concern for industrial workers and for populations living in polluted areas, especially in less developed countries. In the industry, Cd is hazardous both by inhalation and ingestion and can cause acute and chronic intoxications. Cd dispersed in the environment can persist in soils and sediments for decades. When taken up by plants, Cd concentrates along the food chain and ultimately accumulates in the body of people eating contaminated foods. Cd is also present in tobacco smoke, further contributing to human exposure. Further, the most salient toxicological property of Cd is its exceptionally long half-life in the human body. Once absorbed, Cd irreversibly accumulates in the human body, in particularly in kidneys and other vital organs such the lungs or the liver. Acute exposure to Cd in vivo causes dysuria, polyuria, chest pain, fatigue, headache, and hepatooxidative [1]-[3]. Role of this heavy metal was proposed in the formation of advance glycation end products (AGEs) by nonenzymatic reaction [4].

The nonenzymatic reaction between reducing sugars and proteins, known as glycation, has received increased attention in nutritional and medical research. Nonenzymatic glycation is a complex series of reactions between reducing sugars and amino compounds. As the first step of AGEs formation, the free amino groups of proteins in the tissues react with a carbonyl group of reducing sugars, such as glucose, to form glucosamines via a Schiff base by Amadori rearrangement. Both Schiff base and Amadori product further undergo a series of reactions through dicarbonyl intermediates [e.g., glyoxal (GO), methylglyoxal (MG) and 3-deoxyglucosone], to form AGEs. GO and MG, the two major α-dicarbonyl compounds found in the human body, are extremely reactive and readily modify lysine, arginine, and cysteine residues of proteins [5]. Reactive carbonyl compounds such as GO and MG have recently attracted much attention because of their possible clinical significance in chronic and age-related diseases [6]-[8]. For example, MG mediates vascular inflammation in human endothelial cells [9], gastric ulcer [10], and renal disease [11].

In recent years, a number of protein crosslinks have been isolated that are thought involve the MG. MG is a potent protein and nucleic acid modifying agent found in all mammalian systems as a consequence of energy metabolism. MG is produced through spontaneous phosphate elimination from glycolytic pathway

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intermediates. MG levels also respond to signaling events associated with cell death, indicating that anabolic activities for MG production that may be present in mammalian systems as they are in bacteria, although that is yet to be demonstrated. The physiological concentration of MG is thought to range between 256 nM in blood (2.4 µM in diabetics), 1 µM in plasma and 15 µM in urine in healthy human. However, up to 310 µM has been reported where assay systems have quantified reversibly protein-bound methylglyoxal along with unbound. Ninety-nine percent of methylglyoxal is thought exist in reversibly bound state to protein or other biological ligands [12], [13].

The previous study described that the reaction of MG with ceruloplasmin may lead to decreased feroxidase activity in vitro [14]. In addition, the ferritin/MG/lysine system may lead to oxidative DNA damage via the generation of ROS by the Fenton-like reaction of free iron ions released from oxidatively damaged ferritin [15]. Based on previous research, methylglyoxal formation can be accelerated by metals in vitro. The proposed mechanism explained that the metal M^{n+} (e.g., Fe^{2+}, Cu^{2+}, and so on) can catalyze the 2,3-enediol and formed MG and hydroperoxide [16]. The role of Cd in the formation of MG and hydrogen peroxide has not been much studied. Thus, our study aims to measure the formation rate of MG and hydrogen peroxide in the presence of Cd in vitro.

II. Material and Methods

We modify the protein glycation by using Bovine serum albumin as the protein which reacts with high concentration D-glucose. Cadmium which used in this in vitro model is $(CH_3COO)_2Cd$. This research was divided into 4 groups (1 control group and 3 treatment groups). Control: BSA 125 mM D-glucose + 125 mM, P1: + 125 mM D-glucose + 125 mM + $(CH_3COO)_2Cd$ 0.003 mg / L; P2: + 125 mM D-glucose + 125 mM + $(CH_3COO)_2Cd$ 0.3 mg / L P3: + 125 mM D-glucose+125 mM+$(CH_3COO)_2Cd$ 30 mg / L. Solution was incubated at 37 °C and observed on days 2, 4, 6, and 8.

A. Carbonyl Compound Assay

Sample derivatization. Two 1-mg aliquots are needed for each sample to be assayed. Samples are extracted in a final concentration of 10% (w/v) TCA. The precipitates are treated with 500 µL of 0.2% DNPH or 500 µL of 2 M HCl. Samples are incubated at room temperature for 1 h with vortexing at 5-min intervals. The proteins are then precipitated by adding 55 µL of 100% TCA. The pellets are centrifuged and washed three times with 500 µL of the ethanol:ethyl acetate mixture. The pellet is then dissolved in 600 µL of 6M guanidine hydrochloride. The carbonyl content is determined by reading the absorbance at the optimum wavelength 390 nm [17].

B. Methylglyoxals Assay

Methylglyoxals are estimated according to the modified method of Racker [18]. Twenty-five µl of samples was added to 350 µl of DNPH [0.1% DNPH in 2N HCl]. Then to each tube 2.125 ml of distilled water was added. Then it was incubated for 15 minutes at 37^0C. After the incubation 1.5 ml 10% NaOH was added and absorbance was read at 576 nm using spectrophotometer. MG levels are expressed in percent absorbance MG and dicarbonyl absorbance.

C. Hydrogen Peroxide Assay

90 ml of sample, 10 ml of methanol and 900 ml of xylenol orange reagent containing ferrous ions were added successively and absorbance was noted at 560 nm [19].

D. Statistical Analysis

MG and hydrogen peroxide formation were analyzed by first order kinetics equation. Then calculated the constant of a first order kinetic. For analyzing of the data, we used ANOVA and regression correlation test. For all outcomes, a nominal p-value of $p < 0.05$ was considered significant.

III. Results and Discussion

A. The Role of Cd in Methylglyoxal Formation

The average levels of MG produced glucose and protein reactions are presented in Table I.

TABLE I. MG Levels (%) (Mean ±SE) of Various Incubation Times and Levels of Cd

Groups	Times Incubation (day)			
	2	4	6	8
Control	5.155 ± 0,12	5.785 ± 0.47	14.006 ± 3.28	16.097 ± 2.87
+ $(CH_3COO)_2Cd$ 0.003 mg / L	8.333 ± 0.23	19.804 ± 0.95	41.333 ± 3.09	45.323 ± 1.23
+ $(CH_3COO)_2Cd$ 0.3 mg / L	10.417 ± 1.46	18.821 ± 2.33	41.623 ± 2.19	67.432 ± 1.98
+ $(CH_3COO)_2Cd$ 30 mg / L	11.515 ± 2.32	19.545 ± 3.29	39.847 ± 2.21	98.976 ± 4.86

ANOVA test results showed that there were significant differences between treatment groups ($p < 0.05$). First order reaction rate of formation methylglyoxal are presented in Fig. 1.

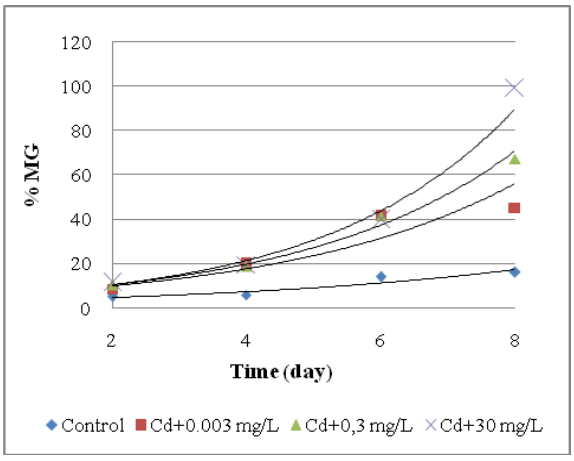

Figure 1. First order kinetics of formation methylglyoxal

Reaction rate constants of MG formation calculated using regression analysis (Table II).

TABLE II. REACTION RATE CONSTANTS (K) OF MG FORMATION

Groups	Methylglyoxal		
	k (day^{-1})	r	p (<0.05)
Control	0.215	0.943	0.047
+ $(CH_3COO)_2Cd$ 0.003 mg / L	0.291	0.955	0.045
+ $(CH_3COO)_2Cd$ 0.3 mg / L	0.320	0.996	0.004
+ $(CH_3COO)_2Cd$ 30 mg / L	0.358	0.993	0.007

Glucose combines a delicate balance between chemical stability and chemical reactivity for metabolism synthesis. It also exists in several structural synthesis, divided based on the conformations: two major forms of pyranose (six-member rings with anomeric carbon at member rings with carbon at position 1, one minor form of furanose (five-member ring), and an open aldehyde. However, the linear aldehyde form is essential intermediate for conversion among these forms. The linear aldehyde glucose is more reactive structure that can bind to the amine group on the protein become glycated protein. In Table II shows that increasing concentrations of Cd has positive effect on the formation rate of glucose. This is consistent with the mechanism proposed by Voziyan *et al* [4], that metals can accelerate the formation of compounds dicarbonyl [e.g., glyoxal (GO), methylglyoxal (MG) and 3-deoxyglucosone].

MG as a side-product of glycolysis consequently arises from an increased flux during hyperglycemia. MG has been postulated to play a role in the development of hypertension [20]. Studies using animal model and cell cultures showed a significant increase in blood pressure to coincide with elevated MG level in plasma and aortic tissues [21]. However, functional links between MG biogenesis and hypertension, in part mediated by ROS and AGEs, have only been documented in rat model but not yet in humans under these conditions.

B. The Role of Cd in Hydrogen Peroxide Formation

The average levels of Hydrogen peroxide produced glucose and protein reactions are presented in Table III.

TABLE III. HYDROGEN PEROXIDE LEVEL (μM) (MEAN ±SE) OF VARIOUS INCUBATION TIMES AND LEVELS OF CD

Groups	Times Incubation (day)			
	2	4	6	8
Control	0.394 ±0.02	0.825 ±0.12	1.524 ±0.13	3.944 ±0.92
+ $(CH_3COO)_2Cd$ 0.003 mg / L	0.502 ±0.01	3.621 ±0.91	6.472 ±0.43	19.218 ±0.75
+ $(CH_3COO)_2Cd$ 0.3 mg / L	0.556 ±0.03	4.213 ±0.88	6.794 ±0.38	35.514 ±0.67
+ $(CH_3COO)_2Cd$ 30 mg / L	0.717 ±0.02	4.805 ±0.67	6.794 ±0.89	52.671 ±0.69

ANOVA test results showed that there were significant differences between treatment groups ($p < 0.05$). First order reaction rate of formation hydrogen peroxide are presented in Fig. 2.

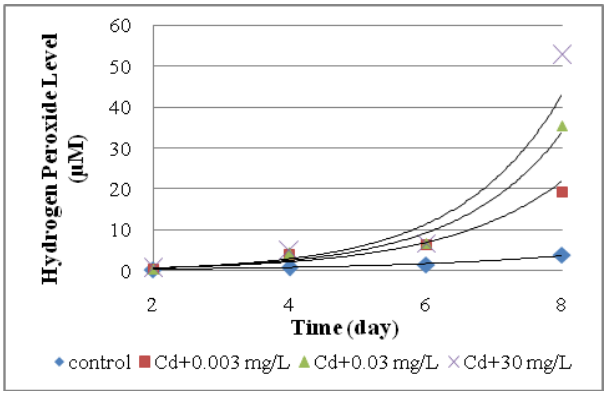

Figure 2. First order kinetics of formation hydrogen peroxide

Reaction rate constants of hydrogen peroxide formation calculated using regression analysis (Table IV).

TABLE IV. REACTION RATE CONSTANTS (K) OF HYDROGEN PEROXIDE FORMATION

Groups	Hydrogen Peroxide		
	k (day^{-1})	r	p (<0.05)
Control	0.376	0.996	0.040
+ $(CH_3COO)_2Cd$ 0.003 mg / L	0.576	0.973	0.027
+ $(CH_3COO)_2Cd$ 0.3 mg / L	0.647	0.977	0.033
+ $(CH_3COO)_2Cd$ 30 mg / L	0.662	0.971	0.029

Environmental stresses are known to induce hydrogen peroxide and other toxic oxygen species production in cellular compartments and result in acceleration of lipid peroxidation and other oxidative damage. Hydrogen peroxide being a strong oxidant that can initiate localized oxidative damage in cells leading to disruption of metabolic function and loss of cellular integrity resulting in senescence promotion. It also changes the redox status of surrounding cells.

Based on the proposed mechanisms by Voziyan et al [4], hydrogen peroxide formed in a phase when conversion of 2,3-enediol to dicarbonyl compound. As in the formation of MG, the hydrogen peroxide concentration increases concordant with levels of Cd.

In human, hydrogen peroxide is produced in many different cell types, including fibroblast, vascular endothelial, smooth muscle, and inflammatory cells . It is known to act as a cellular signaling molecule within blood vessels, and it plays key roles in regulating vascular smooth muscle cell (VSMC) growth, differentiation, migration, and vascular inflammation. Hydrogen peroxide has been shown to cause constriction in a variety of vascular beds under quiescent conditions, and it can induce vasoconstriction in a number of arteries in vitro, including rat aorta, vena cava and pulmonary artery, canine basilar artery, and human placental arteries [22].

This study is similar with a study by Adrover et al [23]. They showed that formation of glycoaldehyde from glycated protein was kinetically happen in first order reaction and the rate constant was 0.33 ±0.03 h^{-1}.

IV. CONCLUSION

We found that there are significant correlation between Cd exposure on the formation of hydrogen peroxide and methylglyoxal ($p < 0.05$) in nonenzymatic glycation of proteins by glucose. The increased Cd level accelerate the formation of methylglyoxal and hydrogen peroxide.

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