International Journal of Biochemistry Research & Review
6(4): 151-159, 2015, Article no.IJBcRR.2015.047
ISSN: 2231-086X

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Changes of Carbonyl Stress Parameters in Rats with Diabetes and Rhabdomyolysis

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Authors' contributions

This work was performed in collaboration between all the authors. Author SS designed the project and supervised the work. Authors KT and IK carried out all laboratories work and performed the statistical analysis. Author KT wrote the manuscript and managed the literature searches. Author SS edited the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBcRR/2015/15161
Editor(s):
(1) Mohamed Fawzy Ramadan Hassanien, Biochemistry Department, Zagazig University, Egypt.
(2) Luisa Di Paola, Chemical and Biochemical Engineering Fundamentals, Faculty of Engineering Università Campus Biomedico, Via Alvaro del Portillo, Roma, Italy.
Reviewers:
(1) Gerald Tomkin, Department of Clinical Medicine, Trinity College Dublin, Dublin, Ireland.
(2) Anonymous, India.
(3) Anonymous, India.
(4) Anonymous, China.
(5) Helen Saada, Radiation Biology Department, National Centre for Radiation Research and Technology, Atomic Energy Authority, Cairo, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=927&id=3&aid=8189

Original Research Article

Received 10th November 2014
Accepted 5th February 2015
Published 20th February 2015

ABSTRACT

Aim: To investigate the changes of carbonyl stress parameters in rats with combined streptozotocin induced diabetes (STZD, hyperglycemia model) and glycerol stimulated rhabdomyolysis (RM, oxidative stress model).
Methodology: RM was induced by glycerol injection and confirmed by changes of heme oxygenase activity, creatine kinase activity, total heme, creatinine, uric acid, urea and bilirubin. STZD was induced by streptozotocin injection and proved by increasing of glucose levels. Carbonyl stress development was determined by total aldehyde levels, TBARS, protein CO group levels, N(6)-Carboxymethyllysine and low-molecular-weight SH groups in blood plasma and liver tissue.
Results: A significant increase of carbonyl stress parameters was recorded in RM and STZD rat groups compared with their relative control levels. In animals subjected to the combined induction of

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RM and STZD, the level of carbonyl stress parameters was lower than that recorded for the STZD and RM groups: total aldehyde levels were decreased in liver resulting in lower TBARS and protein CO group levels. Low-molecular-weight SH groups were increased compared with STZD.
Conclusion: Changes of carbonyl stress parameters indicated the significant role of carbonyl stress in diabetes and in rhabdomyolysis. It was demonstrated that combined stimulation of RM and hyperglycemia led to decreasing of carbonyl stress parameters. It can be suggested that these changes in carbonyl stress parameters can be associated with additional initiation of antioxidant defense systems or some compensatory mechanisms.

Keywords: Carbonyl stress; oxidative stress; diabetes; rhabdomyolysis.

1. INTRODUCTION

Carbonyl stress is the result of the transformation of lipid radicals formed during lipid peroxidation or the consequence of non-enzymatic protein glycosylation [1,2]. The most common reactive aldehydes found in biological systems are malondialdehyde, glyoxal, methylglyoxal, 3-deoxyglucosone, acrolein, etc. Lipid-derivatives aldehydes are more stable than their precursors free radicals and thus can diffuse from the initial site of lipid peroxidation and propagate oxidative injury by acting as toxic messenger [3]. These compounds are electrophilic, and thus are highly reactive with different cellular constituents majority of which are nucleophiles. Such strong nucleophilic sites as thiols, imidasole and hydroxyl groups of biomolecules as well as nitrogen and oxygen atoms in purine and pyrimidine bases are the most attractive targets for electrophilic attacks. Reactive endogenous aldehydes can bind covalently to Lis, His and Cys residues and form crosslinks in proteins that lead to the disruption of their structure and functions [4]. These changes resulted in irreversible molecules modifications and formation of variety adducts named advanced lipoxidation end products (ALEs) and advanced glycosylation end products (AGEs) (Fig. 1). There are pentosidine (cross-link interaction between Arg and Lys), carboxymethyllysine (oxidative degradation of the Amadori products, the reaction of glyoxal with Lys, and by oxidation of both the Schiff base and the Amadori products), carboxyethyllysine (the reaction of methylglyoxal with and Lys), pyraline (the reaction of 3-deoxyglucosone with Lys) [5-6]. Protein carbonyl groups (CO) formation is the other protein modifications, produced by secondary reaction of the nucleophilic side chains of Cys, His, and Lys residues, with aldehydes (4-hydroxy-2-nonenal, malondialdehyde, acrolein) during lipid peroxidation [7-9]. The pathologic effects of AGEs/ALEs are related to their ability to promote oxidative stress and inflammation by binding with cell surface receptors, cross-linking with body proteins (such as collagen), altering their structure and function or modifying blood vessel structure [10,11]. The increasing in the steady-stage concentrations of AGEs and ALEs is related to hypertension, kidney and heart diseases, Alzheimer's and Parkinson's diseases, cataractogenesis, atherosclerosis, diabetes and diabetic secondary complications [12,13].

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion and insulin action or both. The chronic hyperglycemia is associated with long-term damage, dysfunction, and failure of normal functioning of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels [14-16]. People with diabetes showed the risk of the development of acute metabolic complications such as diabetic ketoacidosis or hyperglycaemic hyperosmolar nonketotic coma. RM is common in diabetic patients with hyperosmolarity, caused by hyperglycemia. The hyperosmolal state predisposes to the development of RM [17]. RM, the dissolution of skeletal muscles, is characterized by the leakage of muscle-cell contents, including electrolytes, myoglobin, and other sarcoplasmic proteins into the blood circulation [18]. Myoglobin may release free ferrous (Fe^{2+}) ions, which could induce lipid peroxidation as a result of the generation of hydroxyl radicals via Fenton's reaction [19].

In our previous studies, it has been demonstrated that carbonyl stress plays an important role in diabetes and RM [20-21].

The aim of the present study was to investigate the changes of carbonyl stress parameters in rat experimental models of streptozotocin induced diabetes (STZD, hyperglycemia model) and glycerol stimulated rhabdomyolysis (RM, oxidative stress model) and in combined model of these pathologies.

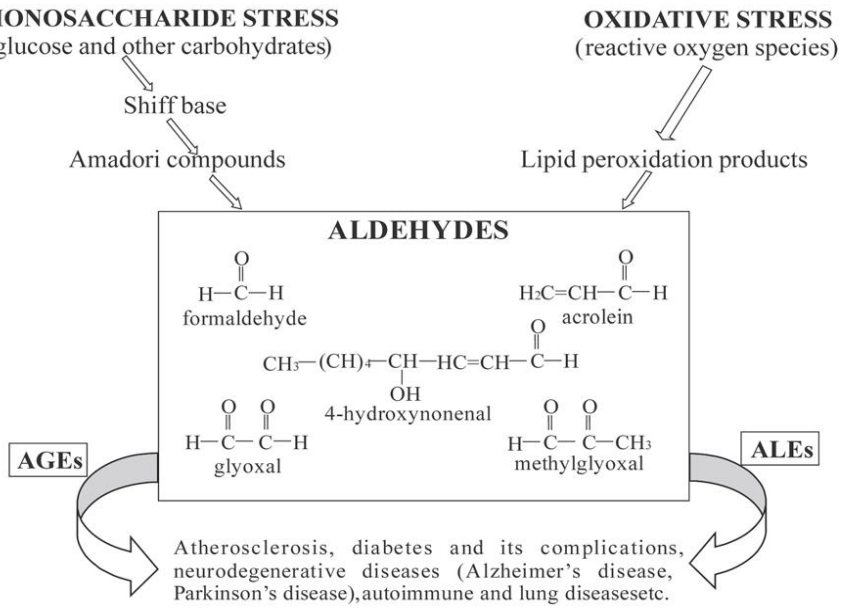

Fig. 1. Formation of reactive aldehydes and advanced glycation and lipoxidation end products in oxidative and carbonyl stress

2. MATERIALS AND METHODS

2.1 Animals and Experiment Design

Experiments were performed on male Wistar rats weighed 180-220 g, which were maintained on a standard vivarium diet. Animals were randomly divided into 8 experimental groups (n=8 per group) as follows: group 1 control, groups 2-6 rhabdomyolysis (RM), group 7 diabetes (STZD), group 8 rhabdomyolysis + diabetes (RM + STZD).

For RM induction animals of 2-6 groups were injected with 50 % glycerol solution into both femoral muscles at dose 10 ml/kg. Animals were decapitated at 1st day (gr.2), 3rd day (gr.3), 4th day (gr.4), 6th day (gr.5), 10th day (gr.6) after glycerol injection.

Modeling of diabetes was performed by using the described technique [22]. For induction of STZD (Sigma, USA) the diabetic groups (gr.7, 8) were injected intraperitoneally with streptozotocin three times: first and third time at a dose of 25 mg/kg in 0.5 ml of citrate buffer (pH 4.5) and second time – 20 mg/kg at weekly interval. 24 h before streptozotocin injection animals were given 1 ml of incomplete Freund's adjuvant intraperitoneally.

One week after last streptozotocin injection, animals in group 8 were injected with glycerol solution and at 10th day after that, animals in both 7 and 8 groups were decapitated.

2.2 Samples Preparation

Blood and liver samples were collected for investigation. The liver was homogenized in Tris-HCl buffer (pH 7.4) with 0.25 M saccharose. Blood and serum were separated by centrifugation at 3000 g for 10 min.

2.3 RM and STZD Parameters Determination

In STZD for the determination of blood glucose using glucometer "Glucofot Plus" (LLC "Diasvit", Ukraine), whole blood was collected from the tail vein one week after last streptozotocin injection.

In RM heme oxygenase (HO) activity was determined in liver by the bilirubin formation [23]. Total heme content was measured by pyridin hemochrome assay. Creatine kinase activity was determined by "Audit Diagnostics" kit (Ireland). Detection of bilirubin HP005.01, creatinine HP014.01, uric acid HP017.01 and urea HP018.01 were performed by "Filicit Diagnistics" kits (Ukraine).

2.4 Carbonyl Stress Parameters Determination

The concentration of aldehydes in liver was determined by Purpald reagent (Sigma, USA) using formaldehyde solutions as the standard [24].

Protein CO group levels were quantified as described by Alamdari et al. [25]. The CO groups in the protein side chains are derivatized to 2.4-dinitrophenylhydrazone by reaction with 2.4-dinitrophenylhydrazine (DNPH). These modified CO groups were determined in plasma and liver by ELISA using rabbit anti-DNP Antibody (Sigma, USA). The absorbance was read spectrophotometrically at 490 nm using μQuant (Biotek, USA).

The formation of MDA, an end product of fatty acid peroxidation, was measured in liver spectrophotometrically at 532 nm (Biotek, USA) by using a thiobarbituric acid reactive substance (TBARS) [26]. 1,1,3,3-tetraethoxypropane was used as the standard.

Low-molecular-weight SH group levels in liver were measured fluorometrically using the fluorochrome *o*-phtalaldehyde (Fluka, Austria) [27]. Excitation wavelength for *o*-phthalaldehyde was 360 nm and emission was detected at 420 nm by FL800 (Biotek, USA). Reduced glutation was used as the standard (Sigma, USA).

N(6)-Carboxymethyllysine (CML) content in blood plasma was measured by using CML ELISA kit E02C0773 (Shanghai BlueGene Biotech CO., LTD, China).

2.5 Determination of Protein Concentration

Total protein concentrations were measured by biuret method with bovine serum albumin as a standard.

2.6 Statistical Analyses

Data were analyzed using MS Excel 2007. All the measurements were done three times. A Student's t-test was used to estimate differences between the groups. All parameters were given as mean±standard deviation. The criterion for significance was $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1 Glycerol Stimulated Rhabdomyolysis Development

It was shown that at the 1st day after glycerol injection total content of free heme increased 10 times as result of hemoproteins, such as myoglobin, release from damaged cells. From 3rd to 10th day of experiment this parameter decreased but at 10th day it was still higher than control. The most sensitive marker of muscle cell damage is plasma creatine kinase, which seems to correlate with the degree of damage incurred. The fast increasing of creatine kinase activity in blood plasma indicated the damage of muscle cells in RM. At the 1st day of experiment this parameter was more than 10-fold higher than control, and then gradually decreased at the same way as heme. Correlation coefficient between these parameters was 0.99 that indicated muscle degradation and intracellular components release in blood. One of the organism protective mechanisms from toxic effects of heme is heme utilization in HO reaction. At 1st – 3rd day after glycerol injection HO activity was 5-6 times higher than in control that indicated induction of this enzyme. As result of HO functioning heme group transforms to bilirubin that led to fast increase of it in blood. At 1st – 3rd day of experiment bilirubin levels were more than 7-fold higher compared with control. Although this parameter is decreasing gradually, and at 10th day of experiment it was 4 times higher than control. The increase of creatinine, urea and uric acid in plasma characterized the impairment of kidneys' excretory function. Changes of these parameters proved the RM development after glycerol injection (Table 1).

3.2 Streptozotocin Induced Diabetes Detection

STZD was confirmed by measurement of blood glucose levels. Glucose levels were 27±8 mmol/l in diabetic groups (7, 8) compared with control 6.5±1.7 mmol/l one week after last streptozotocin injection.

3.3 Changes of Carbonyl Stress Parameters in RM and STZD

In RM (group 4) and in STZD (group 7) carbonyl stress development was revealed by significant increasing of carbonyl stress parameters. At 4th day after glycerol injection total aldehyde level

was increased approximately 2.4 times in liver in STZD and 3 times in RM compared with control (Fig. 2. (A), all results were equal to control). Obtained results indicated the initiation of reactive carbonyl compounds formation that led to further modification of biological molecules.

The TBARS levels were increased in liver 1.6 times compared with control in RM. In STZD this parameter was increased 4.2 times in compared with control. These changes indicated enhancement of lipid peroxidation. MDA, the major lipid peroxidation product, can react with the free amino group of proteins, phospholipids and nucleic acids leading to their structural modification [28]. The TBARS levels in STZD were 2.6 times higher than in RM that indicated the predominance of lipid peroxidation in STZD (Fig. 2. (B)).

The attack of ROS against proteins modifies amino acids; lysine, arginine, proline and histidine residues generating carbonyl moieties, which has been identified as an early marker for protein oxidation and is used as parameter of protein damage. The increase of protein CO group levels 3.4 times in RM and 4.5 times in STZD in plasma compared with control was observed. It was also showed that protein CO group level in STZD was significantly higher than in RM. In liver protein CO group levels were increased in RM approximately 2.8 times and in STZD 3.9 times compared with control. It was also demonstrated the higher level of protein carbonylation in STZD than in RM. These changes can be considered as carbonyl stress consequences. The results are presented at Fig. 2. (C). Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation. Carbonyl stress development led to accumulation of protein modifications such as AGEs. CML is an advanced glycation end product formed on protein by combined nonenzymatic glycation and oxidation (glycoxidation) reactions. CML as result of glyoxal and lysine interaction is known to accumulate in proteins with age and at an accelerated rate in diabetes [29]. It was shown that CML levels were increased 3 times in blood plasma compared with control in STZD. It was shown decreasing of low-molecular-weight SH group level in liver 2.2 times compared with control in STZD. SH groups take place in protecting molecules against electrophiles and free radicals. This is due to their nucleophilicity and the high reaction rate of thiols with free radicals. Obtained results are presented in Table 2.

3.4 Changes of carbonyl stress parameters in combined model of RM and STZD

The high levels of aldehydes and the increasing of the other carbonyl stress parameters confirmed the carbonyl stress development in RM and STZD. Thus, it was suggested that in combined development of RM and STZD it will be an intensive increasing of carbonyl stress parameters. But obtained results were different from expected.

Table 1. Biochemical parameters of the rat blood plasma and liver in rhabdomyolysis (mean±standard deviation, n=8)

Parameters	1 control	2 1st day	3 3rd day	4 6th day	5 10th day
Blood plasma					
Total heme, μmol/l	11±5	120±26*	45±9*	41±10*	34±9*
Creatine kinase activity, μmol/min	32±10	350±95*	184±77*	68±19*	53±18*
Creatinine, μmol/l	62±9	95±9*	79±10	130±18*	243±27*
Uric acid, mmol/l	0.11±0.04	0.19±0.05	0.22±0.05*	0.28±0.07*	0.39±0.11*
Urea, mmol/l	2.4±0.3	4.0±0.5*	6.5±1.0*	8.7±1.6*	8.5±1.7*
Bilirubin, μmol	4.6±0.6	31±4*	34±6*	20±4*	18±4*
Liver					
Heme oxygenase activity, nmol of bilirubin/min/mg of protein	8.1±2.0	47±7*	51±8*	38±5*	22±7*

**p<0.05 compared with control*

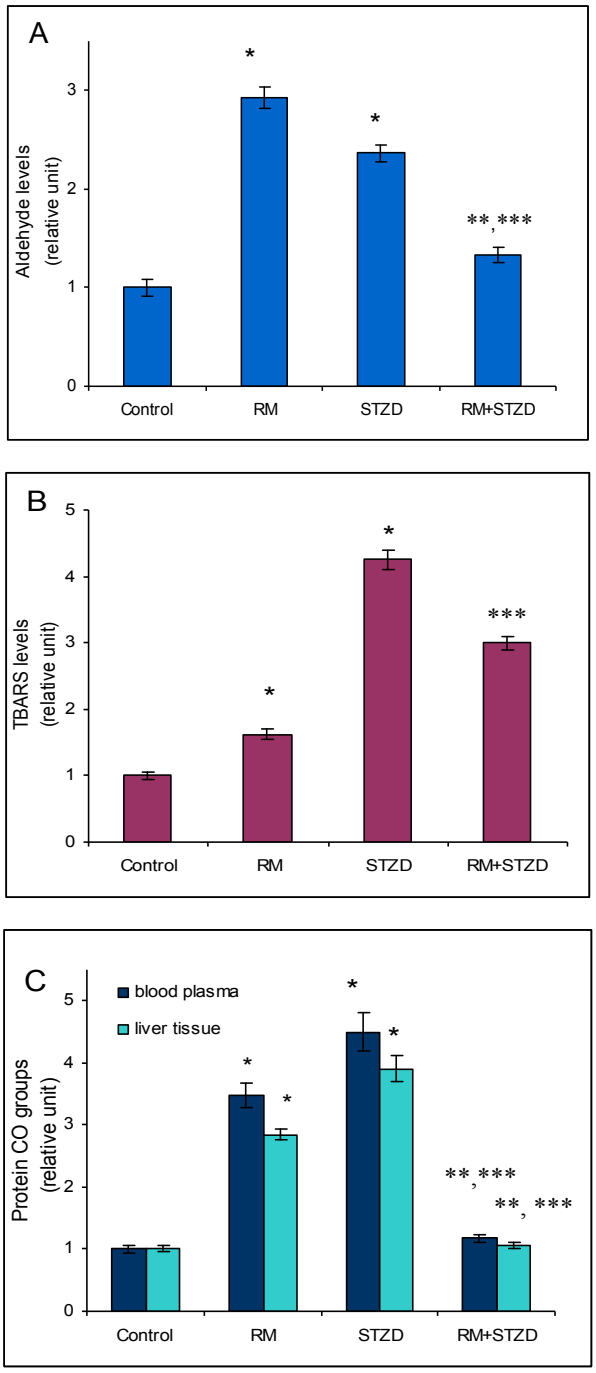

Fig. 2. Changes of carbonyl stress parameters in rats with rhabdomyolysis (RM) and diabetes (STZD): total aldehyde levels in liver (A), thiobarbituric acid reactive substances (TBARS) in liver (B) and levels of protein CO groups in blood plasma and in liver (C), all results were equal to control

**p<0.05 compared with the control, ** p<0.05 compared with the RM (group 4), *** p<0.05 compared with the STZD (group 7)*

Table 2. Carboxymethyllysine and low-molecular weight SH groups measurement in streptozotocin induced diabetes (mean±standard deviation, n=8)

Parameters	Control group 1	Diabetes group 7	Rhabdomyolysis+Diabetes group 8
Blood plasma			
CML, mg/l	0.30±0.05	0.90±0.03*	0.80±0.06
liver			
SH groups, nmol/mg of protein	8.0±0.6	3.6±0.3*	4.5±0.4**

**p<0.05 compared with control; ** p p<0.05 compared with diabetic group 7*

In combined model of RM and STZD, the total aldehyde level in liver was decreased 1.8 times compared with STZD and 2.3 times compared with RM. That resulted in decreasing of TBARS 1.4 times compared with STZD. Compared with RM the decreasing of this parameter was not significant. It was also observed the decreasing of protein CO group levels 3.7 times in plasma and 3.6 times in liver compared with STZD. Compared with RM group this parameter was decrease 2.9 times in blood plasma and 2.6 times. CML level was not significant changed in plasma compared with STZD. Low-molecular-weight SH groups were increased 1.3 times in liver compared with STZD.

It can be assumed that this effect after combined induction of STZD and RM can be explained by initiation of antioxidant defense systems that lead to enhanced elimination of carbonyl compounds. Therefore, the decreasing of carbonyl stress parameters occurs.

Perhaps, the decreasing of carbonyl stress parameters is associated with the initiation of compensatory mechanisms, the action of which is led to reduce the carbonyl damage in the development of these pathologies.

It is known that carbonyl compounds are continuously produced and eliminated. Therefore, the concentration of carbonyls is a steady dynamic parameter. Different influences may change it. Due to some reasons the steady-state carbonyl concentration is acutely or chronically increased leading to oxidative modification of cellular constituents. It has been shown that under pathology, the concentration of carbonyl compounds sharply increases. Long pathological state such as diabetes leads to transition the steady-state carbonyl concentration to chronic state, which is lower than previous as result of the protective mechanisms depletion. Changes of other parameters confirm its dependence on concentration of carbonyl compounds.

In any case, these mechanisms are not established and required further study using more specific markers that will allow the better understanding of the mechanism of carbonyl stress participation in the development of different pathological processes.

4. CONCLUSION

The present study showed that changes of main carbonyl stress parameters such as total aldehyde level, TBARS, protein CO group levels, low-molecular-weight SH group levels and concentration of CML in rat liver tissue and blood plasma indicated the increasing of carbonyl stress in RM and in STZD development. It was shown that combined stimulation of RM and STZD led to significant decreasing of carbonyl stress parameters. It can be suggested that these changes of carbonyl stress parameters can be associated with additional initiation of antioxidant defense systems or some compensatory mechanisms of organism. Such mechanisms are needed to be investigated for further understanding of these processes.

ETHICAL APPROVAL

All *in vivo* experiments were conducted in accordance with the international principles of the European Convention for protection of vertebrate animals, used for experimental and other scientific purposes" (Strasburg, 1985). Rules of work with laboratory animals in Ukraine are regulated by the Law of Ukraine № 3447-IV "On Protection of Animals Against Cruel Treatment" (2006) and "General ethical principles of animal experimentation", proceedings of the 1st National Congress on bioethics, Kyiv, National Academy of Science of Ukraine; 2001.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
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