BIOPHYSICS AND BIOCHEMISTRY

Effect of Alloxan on Spontaneous Lipolysis and Glutathione System in Isolated Rat Adipocytes

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In vitro experiments on cultured adipocytes from epididymal adipose tissue showed that addition of alloxan (0.5-10.0 mmol/liter) to the incubation medium induced the development of oxidative stress accompanied by an increase in the concentration of reactive oxygen species, TBA-reactive substances, and lipid hydroperoxides in cells. The redox state of adipocytes changed significantly under these conditions, which was associated with a decrease in the amount of reduced tripeptide, an increase in the content of glutathione disulfide, and a decrease in the reduced/oxidized glutathione ratio. The development of oxidative stress in adipocytes was accompanied by activation of spontaneous lipolysis, which probably plays an important role in the mechanisms of insulin resistance.

Key Words: *adipocytes; alloxan; oxidative stress; lipolysis; glutathione*

Type 1 and 2 diabetes mellitus is accompanied by oxidative stress, which results in accumulation of lipid peroxidation products in the blood and tissues and inhibition of the antioxidant defense system (primarily of the enzymatic compartment) [1,4,5]. The diabetogenic effect of alloxan is determined by the ability of this drug to generate toxic free radicals selectively damaging β-cells in the islets of Langerhans [13]. Alloxan is oxidized into dialuric acid in cells [13], which is accompanied by generation of the superoxide anion radical from oxygen. The interaction of this radical with alternating-valence metal ions leads to the formation of hydroxyl radical. The hydroxyl radical activates free radical oxidation of polyunsaturated fatty acids in cell membrane phospholipids, causes oxidative modification of proteins (including those involved in hormone signal transduction), and damages DNA molecules [3]. Reactive radicals and LPO products have an adverse effect not only on β-cells in the islets of Langerhans, but also on cells in other organs and tissues. However, the influence of these compounds on adipose tissue remains unknown.

Little is known about the glutathione system and intensity of lipolysis in adipose tissue during alloxan-induced diabetes. We studied the *in vitro* effect of alloxan in various concentrations on lipolytic activity, glutathione content, and redox potential of isolated adipocytes from rat epididymal adipose tissue.

MATERIALS AND METHODS

Experiments were performed on adipocytes from epididymal adipose tissue of male outbred rats (n=16) weighing 210±25 g. The animals were obtained from the Rassvet nursery (Tomsk). The research was conducted according to the Rules of Studies with Ex-

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periments Animals (Order of the Ministry of Health of USSR, No. 755, 12.08.1977), Federal Act on the Protection of Animals (01.09.1997), and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (1986). The rats were anesthetized with CO_2 asphyxia. Adipocytes were isolated from epididymal adipose tissue with collagenase (Sigma Aldrich) [12]. Adipocyte concentration in the suspension was adjusted to 10^6 cells/ml with a buffer. Cell viability was evaluated by trypan blue staining (Serva). The percentage of living cells was at least 95%.

Adipocytes (10^6 cells/ml) were incubated with alloxan in final concentrations of 0.5, 1.0, 5.0, and 10.0 mmol/liter [8] in a CO_2 incubator (Sanyo). Incubation was performed in Krebs–Ringer buffer containing 5 mmol/liter glucose and 2% BSA (V-fraction) for 3 h. After incubation, the cells were separated by centrifugation in a Biofuge Primo R centrifuge (Thermo) at 200*g* for 1 min.

The degree of lipolysis was estimated enzymatically from glycerol concentration in the adipocyte incubation medium [15].

The intensity of peroxidation in adipocytes was evaluated from the content of lipid hydroperoxides (FOX-2 method) [6] and concentration of TBA-reactive substances (fluorescence assay). Fluorescence was measured on a Fluorat-02 biological fluid analyzer (Lyumeks). The excitation maximum of a trimethine complex and fluorescence maximum were set at 515 and 553 nm, respectively.

The antioxidant defense system of adipocytes was studied by measuring the content of total, reduced, and oxidized glutathione (cyclic method [2]) after protein precipitation with 5% sulfosalicylic acid.

A special series was performed to study the effect of alloxan on the concentration of reactive oxygen species (ROS). The cells were preincubated with a fluorescent probe dichlorofluorescin diacetate (5 μmol/liter; Sigma Aldrich). Adipocytes were washed by 3-fold centrifugation (200*g*, 3×1 min). Fluorescence was measured on a Rotor Gene 6000 device at the excitation and emission maxima of 470 and 510 nm, respectively. The measurements were conducted 3 h after alloxan addition. The results were expressed in arbitrary units of fluorescence per 10^6 adipocytes.

The conformity of the experimental samples to normal distribution was verified using the Shapiro–Wilk test. The samples did not conform to the normal distribution at $p<0.001$. Hence, the data in Tables 1 and 2 are presented as the median (Me) and upper and lower quartiles (Q_1-Q_3). The significance of differences between the samples was estimated by nonparametric Mann-Whitney test and Kruskal–Wallis test for small samples. The differences were significant at $p\leq0.05$.

RESULTS

Incubation of adipocytes with alloxan in diabetogenic doses (1.0-10.0 mmol/liter) was followed by the development of oxidative stress. Alloxan in a concentration of 5.0 mmol/liter *in vitro* significantly ($p\leq0.05$) increased fluorescence of dichlorofluorescin diacetate-loaded adipocyte suspension (a probe reacting with ROS). The degree of probe fluorescence increased with increasing the concentration of alloxan in the

TABLE 1. Effect of Alloxan on the Contents of Glycerol, ROS, Lipid Hydroperoxides, and TBA-Reactive Substances in Isolated Rat Adipocytes (Me (Q_1-Q_3))

Group		Glycerol, μmol/10^6 cells	ROS, arb. units/10^6 cells	Lipid hydroperoxides, nmol/10^6 cells	TBA-reactive substances, nmol/10^6 cells
Control (intact adipocytes, *n*=8)		2.4	3.7	52.8	4.2
		(2.2-2.7)	(3.2-4.3)	(48.5-57.2)	(3.8-4.6)
Adipocytes incubated with alloxan, mmol/liter (*n*=8)	0.5	3.0*	4.5	58.9	4.9
		(2.5-3.6)	(4.1-4.8)	(52.3-65.5)	(4.1-5.4)
	1.0	3.7**	5.4	82.3*	6.6
		(3.5-4.1)	(4.9-5.9)	(78.2-86.4)	(6.2-7.1)
	5.0	4.3**	8.5*	90.9**	9.5**
		(4.1-4.7)	(7.6-8.9)	(84.5-97.4)	(9.3-9.7)
	10.0	3.7**	15.9**	98.8**	12.3**
		(3.3-4.2)	(12.4-19.5)	(96.5-101.1)	(11.5-13.5)

Note. Here and in Table 2: *$p\leq0.05$, **$p\leq0.01$, and $^+p<0.05$ compared to the control.

incubation medium, which attested to activation of intracellular ROS generation in adipocytes (Table 1).

The increased generation of ROS in adipocytes after treatment with alloxan is followed by activation of peroxidation of unsaturated phospholipids in biological membranes. This conclusion was derived from the increase in the concentration of intermediate and secondary LPO products. The concentration of lipid hydroperoxides increased by 1.6 times in the presence of 1 mmol/liter alloxan ($p \leq 0.05$). Accumulation of TBA-reactive substances increased by 2.3 times over 3-h incubation with alloxan in a concentration of 5.0 mmol/liter ($p \leq 0.01$; Table 1).

Alloxan caused a decrease in the total glutathione content in isolated adipocytes. The effect became more pronounced with increasing the concentration of this prooxidant in the incubation medium (Table 2). Activation of LPO contributes to a decrease in the total glutathione content. These changes are mediated by the following mechanism: inhibition of *de novo* glutathione synthesis; and consumption of this peptide in the glutathione peroxidase and glutathione transferase reactions. Glutathione peroxidase generates oxidized glutathione during elimination of hydroperoxides. The interaction of oxidized glutathione with proteins leads to the formation of mixed disulfides. The glutathione transferase reaction is followed by glutathione conjugation with end-products of LPO and contributes to the regeneration of lipid hydroperoxides [1]. Therefore, the alloxan-induced decrease in the total glutathione content is mainly related to variations in the amount of reduced glutathione (Table 2).

The decrease in the concentration of reduced glutathione, activation of ROS generation, and increase in the content of lipid peroxidation products in isolated adipocytes reflect a decrease in the redox potential of the glutathione system and development of severe oxidative stress in cells under the influence of alloxan. The reduced/oxidized glutathione ratio determines the redox state of cells. The decrease in this parameter serves as one of the criteria for oxidative stress [1]. Moreover, the reduced/oxidized tripeptide ratio plays an important role in the hormonal regulation of lipid metabolism in adipocytes [9].

The intensity of spontaneous lipolysis was studied during incubation of isolated adipocytes with alloxan in various concentrations. Under conditions of oxidative stress, glycerol concentration in the adipocyte incubation medium increased significantly with increasing the concentration of prooxidant ($p \leq 0.01$; Table 1). Activation of LPO in adipocytes is probably followed by disintegration of the phospholipid bilayer on the surface of lipid drop, which increases availability of triacylglycerols for lipase and promotes activation of spontaneous lipolysis.

Hormone-sensitive triglyceride lipase and perilipin are the major factors for regulation of lipolysis in adipocytes [7]. Hormone-sensitive triglyceride lipase is diffusely distributed in the cytosol of non-stimulated cells, while perilipin covers the surface of lipid drops. It prevents hydrolysis of triacylglycerol molecules with hormone-sensitive triglyceride lipase [10]. Hormonal stimulation (*e.g.*, with catecholamines) leads to an increase in intracellular cAMP concentration and activation of protein kinase A, which phosphorylates hormone-sensitive triglyceride lipase and causes its translocation to the surface of lipid drops [11,14]. Protein kinase A phosphorylates perilipin, which modifies the surface structure of lipid drops and contributes to binding of hormone-sensitive triglyceride lipase. The two

TABLE 2. Effect of Alloxan on the Contents of Total Glutathione, Reduced Glutathione, and Oxidized Glutathione in Isolated Rat Adipocytes (Me (Q_1-Q_3)

Group		Total glutathione, nmol/10^6 cells	Reduced glutathione, nmol/10^6 cells	Oxidized glutathione, nmol/10^6 cells	Reduced/oxidized glutathione ratio
Control (intact adipocytes, *n*=8)		44.2	40.0	5.2	7.8
		(41.8-46.7)	(37.1-42.0)	(4.7-5.6)	(7.2-8.0)
Adipocytes incubated with alloxan, mmol/liter (*n*=8)	0.5	38.9+	34.9	5.5	6.4**
		(37.1-40.7)	(31.9-37.9)	(5.2-5.8)	(6.0-6.9)
	1.0	33.9**	27.8**	6.2**	4.5**
		(32.5-35.4)	(26.6-29.0)	(6.0-6.4)	(4.2-4.8)
	5.0	28.5**	21.7**	6.7**	3.3**
		(27.4-35.4)	(20.9-22.6)	(6.5-6.9)	(2.9-33.6)
	10.0	23.6**	16.4**	7.2**	2.3**
		(27.8-24.4)	(15.7-17.1)	(7.1-7.4)	(2.0-2.5)

simultaneous processes (phosphorylation and activation of hormone-sensitive triglyceride lipase and perilipin protein) are followed by intensive lipolysis [10,14].

Our results indicate that alloxan in various concentrations impairs the redox state of the glutathione system and increases the degree of spontaneous lipolysis in rat adipocytes. These changes become more pronounced with increasing the concentration of the prooxidant in the incubation medium of epididymal adipocytes.

Alloxan-induced activation of spontaneous lipolysis in rat adipocytes probably contributes to an increase in the content of free fatty acids and blood plasma during diabetes. The increase in the content of free fatty acids and progression of oxidative stress can be followed by the development of insulin resistance [7].

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