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ALLOXAN-INDUCED ALTERATIONS IN COMPOSITION AND DYNAMICS OF RED BLOOD CELL MEMBRANES

I. EFFECT OF ALLOXAN ON INTACT RED BLOOD CELLS AND ISOLATED ERYTHROCYTE MEMBRANES

CEZARY WATAŁA,* AGATA BUDZIEJEWSKA and ZOFIA JÓŹWIAK
Chair of Biophysics, University of Łódź, Łódź, Poland

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Abstract—Changes of dynamics and chemical composition in membranes of intact red blood cells and isolated erythrocyte membranes treated with alloxan were investigated in order to assess whether alloxan-induced generation of active forms of oxygen may be critical for erythrocyte destroying. *In vitro* incubation of native red blood cells or prepared erythrocyte membrane ghosts with various concentrations of alloxan gave rise both to levels of membrane TBA-reacting substance and lipid membrane microviscosity both in the deeper and surface regions of lipid bilayer, as evidenced by fluorescence polarization technique. The amount of membrane phospholipid decreased upon alloxan action and that of membrane cholesterol remained rather unchangeable, thus resulting in significant elevation of membrane cholesterol : phospholipid (C : PL) ratio. Both time course and concentration effect of alloxan were found to change exponentially with the different rates of the reaction. There was a linear correlation between 1,6-diphenylhexatriene-1,3,5 (DPH) and 1-anilinonaphthalene-8-sulfonate (ANS) anisotropy coefficients and C : PL ratio (respectively $r = 0.697$ and $r = 0.580$) as well as TBARS levels ($r = 0.386$ for r_{DPH} and $r = 0.324$ for r_{ANS}), thus implying the possible effect of membrane dialdehydes on bilayer components immobilization. Regression coefficients significance testing showed reaction rates of TBARS and C : PL changes to be significantly parallel, contrary to those of fluorescence anisotropy coefficients assessing considerably slower dynamics of alloxan-induced changes. The relevance of changes induced by alloxan in isolated erythrocyte ghosts and intact red blood cells and the compatibility of the present results with several previous studies support the widespreading idea pointing the cell membrane as a main target of damage during alloxan action.

The chemical agent alloxan has been used extensively for the inducing of an experimental model of diabetes mellitus in animals, as it exhibits highly selective cytotoxicity for the pancreatic beta cells [1, 2].

Several investigators consistently suggested that diabetogenic action of alloxan is mediated by the generation of toxic oxygen species [2–4].

Experimentally induced diabetes, provoked by the administration of alloxan-dialurate redox couple, exhibits featuring of the metal-catalyzed Haber–Weiss reaction as the mechanism for the destruction of pancreatic beta cells [1, 3].

The present knowledge, which is far from being exhaustive, indicates that all the prior studies on the mode of action are compatible with the view of selective toxicity of this drug to beta cells and attributable to coincidence of rapid accumulation of alloxan in pancreatic cells and their equisitive sensitivity to peroxides [5–7].

None of the findings detracts from the view that alloxan eventually leads to alterations of the properties of islet cell plasma membranes [1].

The pharmacological nature and partition of alloxan in redox cycling reactions notwithstanding, its mode of action may be sufficiently general for different types of cells and tissues by similar mechanism [8].

* Address all correspondence to: Cezary Watała, Chair of Biophysics, University of Łódź, ul. Banacha 12/16, 90-237 Łódź, Poland.

Both *in vitro* and *in vivo* numerous free radicals scavengers protect against alloxan-induced experimental diabetes [6], and the characteristic of such protection points to the cell membrane as a main target of damage [9].

However, the action of alloxan on other types of cells was poorly investigated. Hence, a study was undertaken to follow the alterations of membrane fluidity and lipid composition in membranes of erythrocytes, given their continuous exposure to alloxan.

MATERIALS AND METHODS

Fasting venous blood, drawn on acid citrate dextrose solution, was obtained by venipuncture from eleven healthy volunteers aged 19–47 years (mean ± 1 SD; 29.53 ± 7.30) and recruited from the Central City Blood Bank.

Erythrocytes, separated from plasma and buffy coat of leucocytes, were washed four times within the subsequent hour. Aliquots of red blood cells were subjected to a moderate hemolysis procedure according to Marchesi and Palade [10] in order to obtain erythrocyte membrane ghosts. Membrane ghosts preparations were resuspended in 0.14 M saline to membrane protein concentration of 2.5 mg protein/ml [11]. Suspensions of red blood cells of hematocrit 50% were incubated with 0.14 M saline alloxan solution at final alloxan concentrations of

1 mg/ml and 2 mg/ml, parallely with control samples without alloxan for 4 hr.

In order to estimate the optimal incubation conditions (time course and alloxan concentration) native erythrocytes (hematocrit 50%) were incubated without alloxan for ½, 1, 2, 4, 6, 12, 24 and 48 hr at 37°, and additionally 50% suspensions of red blood cells were incubated for 4 hr with alloxan at the range of concentrations of 0.5–20 mg/ml.

Time intervals of 4 hr and alloxan concentrations of up to 2 mg/ml were selected for the further study, as inducing trace hemolysis not exceeding 1% with respect to control.

In order to testify the influence of various alloxan concentrations on isolated erythrocyte membranes, the membrane ghost preparations were subjected to alloxan action at four concentration variants: 0.154, 0.308, 0.615 and 0.923 mg of alloxan per 1 mg of membrane protein. The time course of the reaction was investigated by incubating the samples with the highest alloxan concentration over 1, 2 or 4 hr, parallely with relevant control samples incubated without alloxan. All the samples, supplied with trace amounts of streptomycin (20 μg per 1 ml of incubation medium), were incubated in sterile conditions at 37° and kept in darkness.

After the incubation erythrocytes were centrifuged and washed twice to remove the bulk of degraded and active alloxan, the first supernatant being collected and used to determine alloxan-induced red blood cell hemolysis; after that erythrocyte membranes were prepared and treated as above mentioned.

Membrane-bound hemoglobin was expressed as a percentage of the total membrane protein. The degree of post-incubation hemolysis was counted as the multiplicity of the A_{542} value determined for the control sample, assuming mean control hemolysis equal to 1.0 and standard deviation equal to sd = $SD_{[abs]}/\bar{x}_{[abs]}$, where the factors indexed [abs] assess parameters estimated for populations of control absolute values.

The amount of TBA-reacting aldehyde material attached per 1 mg of membrane and hemolysate proteins were determined by the procedure of Kobayashi *et al.* [12] and Placer *et al.* [13], respectively.

Membrane chloesterol and phospholipid levels were estimated according to Babson *et al.* [14] and Vaskovsky *et al.* [15] respectively, after the prior lipid extraction [16].

Fluorescence measurements. A reproducible and simple fluorescence polarization technique was employed to monitor alterations of erythrocyte membrane fluidity by the performance of two different fluorescence probes. 1,6-diphenylhexatriene-1,3,5 (DPH),† which is sensitive enough to monitor even faint changes in fluidity of lipid domains, was used to trace lipid fluidity alterations of the hydrocarbon core [17], whereas the second probe, 1-anilionaphthalene-8-sulfonate (ANS), locates predominantly near membrane surface, interacting with charged phospholipid groups [18].

The anisotropic orientation of both the probes used reflects the degree of the order of the membrane lipid molecule and as such lipid fluidity of the two different bilayer regions [17, 18].

Fluorescence polarization of DPH and ANS embedded into the membrane lipid region was measured upon excitation with polarized monochromatic light, and steady-state fluorescence anisotropy was determined by the emission intensities through an analyzer oriented parallely or perpendicularly to the direction of polarization of the excitation light. Accordingly, anisotropy values were calculated as follows:

$$r = (I_{VV} - I_{VH}G)/(I_{VV} + 2I_{VH}G),$$

where I_{VV} and I_{VH} represent the vertical and horizontal orientation of the electric vector or excitation and emission, respectively. A gratifying factor $G = I_{HV}/I_{HH}$ refers to a correction for the inequality of the detection system to horizontally and vertically polarized emission [18].

Fluorescence spectra were recorded at 25°, on a laboratory-made photon-counting spectrofluorometer equipped with thermostated cell holders. DPH 10^{-3} M tetrahydrofurane solution and ANS 5×10^{-3} M Tris–buffer solution (50 mM, pH 7.0) were added to 100 μg protein/ml sample of erythrocyte ghosts to final DPH and ANS concentrations of 2×10^{-6} M and 2.5×10^{-5} M, respectively. In each case the sample was excited at 366 nm through a monochromator with band-width set at 4 nm, the emission being recorded through a cutoff 4 M $NaNO_2$ filter and additional interference filters: 427 nm for DPH and 480 nm for ANS (VEB Carl Zeiss Jena, GDR).

Statistical elaboration. All the resultant values are expressed as the mean of individual means of triplicates. Normal distribution of the variables was tested by means of a Shapiro–Wilk's procedure, and significance was calculated either by means of Student's *t*-testing and variance analysis or by Mann–Whitney–Wilcoxon testing and Kruskal–Wallis one-way analysis by ranks for all the cases not necessarily assessing normal distribution (values of significance denoted by asterisks).

Significance of alloxan-induced changes in intact red blood cells is given in three comparison variants for each parameter, where numericals 1, 2 and 3 are attributed to the comparison of control with alloxan concentration of 1 mg/ml, 2 — the comparison between control and 2 mg/ml solution, 3 — between concentrations 1 mg/ml and 2 mg/ml, respectively.

Significance of changes in the series of experiments with isolated erythrocyte ghosts at each of the performed alloxan concentrations is given with respect to control and as the result of variance analysis (A). All the partial resultant values in temporal variant of experiment were calculated as a percentage change with respect to the control value (sample incubated at a given time without alloxan), accepted to be equal to 100%, and assuming control standard deviation as equal $(SD/\bar{x}) \times 100\%$.

Partial correlation analysis was performed as the

† Abbreviations used: ANS, 1-anilinonaphthalene-8-sulfonate; C, cholesterol; DPH, 1,6-diphenylhexatriene-1,3,5; ESR, electron spin resonance; PL, phospholipids; r_{ANS} and r_{DPH}, fluorescence anisotropy of ANS and DPH respectively; TBARS, 2-thiobarbituric acid reacting substance.

Table 1. Chemical characteristics of erythrocyte membrane ghosts isolated from intact red blood cells subjected to alloxan action

Sample parameter	Control (11)	Alloxan concentration (mg/ml)		Significance
		1 (11)	2 (11)	
Trace haemoglobin (%)	2.50 ± 0.56	4.91 ± 0.87	8.59 ± 2.10	1 0.0001 2 0.0001 3 0.0001
Post-incubation hemolysis (%)	1.00 ± 0.06	1.24 ± 0.18	1.59 ± 0.45	1 0.0001 2 0.0001 3 0.013
Membrane TBARS ($\times 10^3$) [A_{532}/mg protein]	137 ± 35	160 ± 29	182 ± 47	1* 0.039 2* 0.028 3* 0.121
Hemolysate TBARS ($\times 10^3$) [pmol/mg Hb]	90 ± 16	96 ± 16	100 ± 25	1 0.170 2 0.123 3 0.328
PL ($\times 10^3$) [mmol/g protein]	997 ± 112	759 ± 46	676 ± 95	1 0.0001 2 0.0001 3 0.008
C ($\times 10^3$) [mmol/g protein]	764 ± 68	737 ± 64	735 ± 71	1 0.175 2 0.180 3 0.473
C:PL ($\times 10^3$)	776 ± 81	924 ± 83	1046 ± 77	1 0.0001 2 0.0001 3 0.001

Number of samples examined is given in parentheses.
Each value represents mean ± 1 SD.
Incubation time: 4 hr.

Table 2. Steady-state anisotropy of 1,6-diphenyl-hexatriene-1,3,5 and 1-anilinonaphthalene-8-sulfonate in membranes of intact erythrocytes treated with alloxan

Sample parameter	Control (11)	Alloxan concentration (mg/ml)		Significance
		1 (11)	2 (11)	
r_{DPH} ($\times 10^3$)	180 ± 7	186 ± 8	191 ± 7	1 0.054 2 0.001 3 0.067
r_{ANS} ($\times 10^3$)	206 ± 12	214 ± 14	224 ± 14	1 0.083 2 0.002 3 0.055

Number of samples examined is given in parentheses.
Each value represents mean ± 1 SD.
Incubation time: 4 hr.

measure of the variables' interdependence.

Iterative nonlinear regression procedure and regression coefficients significance testing were employed to estimate the parallel character of the various parameter alterations found in erythrocyte membranes given their exposure to various alloxan concentrations and the various time of incubation.

RESULTS

Alloxan action on intact red blood cells.

Tables 1 and 2 present chemical characteristics and steady-state anisotropy values for the membranes isolated from red blood cells subjected to alloxan action. As it might be expected both the amount of membrane trace hemoglobin and the degree of alloxan-induced hemolysis are significantly elevated in membrane preparations of alloxan-treated erythrocytes. Similarly proned changes may be noticed as to TBARS level and anisotropy values, however, the alterations of hemolysate TBARS, though elevated, are poorly significant.

Distinctly marked correlation was established between anisotropy coefficients and C:PL ratio ($r = 0.697$ for r_{DPH} and $r = 0.580$ for r_{ANS}), and it was significantly lower in the case of membrane TBARS level ($r = 0.386$ and $r = 0.324$, respectively; the critical value was 0.453 at $P < 0.01$).

Alloxan action on isolated erythrocyte membrane ghosts.

Significant elevation of membrane TBARS level as well as concomitant increase of both DPH and ANS anisotropy values in membrane ghosts given their exposure to alloxan were found (Tables 3 and 4). Conversely, the membrane phospholipid amount lowers as alloxan concentration increases. Regression coefficients' significance testing showed relative alterations vs alloxan concentration in the case of TBARS level and C:PL ratio to be significantly parallel ($P < 0.380$), whereas quite different kinetics of changes were found when comparing C:PL ratio with fluorescent probes anisotropy alterations ($0.02 > P > 0.01$ for r_{DPH} and $P < 0.01$ for r_{ANS}). The rate of relevant phospholipid

Table 3. Chemical composition and lipid fluidity of erythrocyte membranes treated with various doses of alloxan

Alloxan concentration (mg/ml)	TBARS (A_{532}/mg protein) ($\times 10^3$)	C (mmol/g protein) ($\times 10^3$)	PL (mmol/g protein) ($\times 10^3$)	C:PL ($\times 10^3$)	r_{DPH} ($\times 10^3$)	r_{ANS} ($\times 10^3$)
0	200 ± 27	669 ± 62	1080 ± 86	721 ± 96	207 ± 21	188 ± 15
0.385	228 ± 32	658 ± 64	900 ± 99	829 ± 85	224 ± 14	194 ± 12
0.770	244 ± 42	657 ± 68	795 ± 119	885 ± 56	225 ± 14	202 ± 8
1.540	279 ± 47	679 ± 69	694 ± 109	1016 ± 79	238 ± 22	210 ± 9
3.850	306 ± 38	675 ± 59	637 ± 84	1145 ± 112	242 ± 10	217 ± 13
Significance	1*: 0.023	1: 0.343	1: 0.0001	1: 0.006	1: 0.019	1: 0.156
	2*: 0.018	2: 0.335	2: 0.0001	2: 0.0001	2: 0.014	2: 0.006
	3*: 0.003	3: 0.362	3: 0.0001	3: 0.0001	3: 0.001	3: 0.0001
	4*: 0.0001	4: 0.409	4: 0.0001	4: 0.0001	4: 0.0001	4: 0.0001
	*A**: 0.005	*A*: 0.379	*A*: 0.0001	*A*: 0.0001	*A*: 0.004	*A*: 0.001

Number of samples examined is 11. Each value represents mean ± 1 SD. Incubation time: 2 hr.

Table 4. Chemical composition and lipid fluidity of erythrocyte membranes incubated with alloxan

Incubation time (hours)	TBARS (A_{532}/mg protein)	C (mmol/g protein)	PL (mmol/g protein)	C:PL	r_{DPH}	r_{ANS}
Control (33)	(227 ± 17) $\times 10^{-3}$	(696 ± 81) $\times 10^{-3}$	(1159 ± 166) $\times 10^{-3}$	(591 ± 41) $\times 10^{-3}$	(229 ± 6) $\times 10^{-3}$	(193 ± 12) $\times 10^{-3}$
1 (11)	115.1 ± 9.8	101.7 ± 16.2	76.1 ± 13.7	144.4 ± 13.2	103.2 ± 1.2	109.1 ± 4.7
2 (11)	131.7 ± 14.2	107.2 ± 22.8	67.1 ± 13.9	158.1 ± 19.5	105.3 ± 2.5	109.6 ± 3.7
4 (11)	145.0 ± 18.0	108.9 ± 14.0	58.0 ± 9.2	199.9 ± 10.2	105.3 ± 1.0	113.2 ± 4.3
Significance	1: 0.0001	1: 0.368	1: 0.0001	1: 0.0001	1: 0.001	1: 0.0001
	2: 0.0001	2: 0.139	2: 0.0001	2: 0.0001	2: 0.0001	2: 0.001
	3: 0.0001	3: 0.038	3: 0.0001	3: 0.0001	3: 0.0001	3: 0.0001
	A: 0.0001	*A*: 0.232	*A*: 0.0001	*A*: 0.0001	*A*: 0.0001	*A*: 0.0001

Number of samples examined is given in parentheses.
Each value represents mean ± 1 SD.
Absolute values of control samples are given at the top.
Significance of changes at each incubation time is given with respect to control (100%) and as the result of variance analysis (*A*).
Alloxan concentration: 3.85 mg/ml.

diminution is markedly symmetrical with regard to the above discussed parameters. When analyzing the rate of membrane constituent affection in the time course of the reaction, similarly proned biases may be observed to occur. As evidenced by regression coefficients' significance testing, drastic and most dynamic changes of the C:PL ratio, and even TBARS level, are not accompanied by equally intensified changes in membrane fluidity ($P < 0.002$ and $P < 0.003$ for C:PL vs r_{DPH} and r_{ANS}, respectively; $P < 0.003$ and $P < 0.006$ for TBARS vs r_{DPH} and r_{ANS}, respectively).

As might be predicted, a significant correlation was established between DPH and ANS anisotropy coefficients and C:PL ratio ($r = 0.622$ and $r = 0.706$), as well as moderate reverse correlation with membrane phospholipid level ($r = -0.369$ and $r = -0.662$, respectively). Positive correlation of DPH and ANS anisotropy with membrane TBARS is noteworthy ($r = 0.647$ and $r = 0.584$, respectively), thus implying the possible effect of dialdehydes on membrane components immobilization.

DISCUSSION

The results clearly show that, under the conditions stated, the diabetogenic agent alloxan induced drastic degradation of membrane phospholipids with simultaneous faint destroying of cholesterol. It seems reasonably certain that the significant diminution of membrane phospholipid and unchanged level of membrane cholesterol imply just as pronounced alterations of membrane fluidity. These changes of membrane lipids resulted in considerable increase of steady-state fluorescence anisotropy of both employed fluorescent probes, indicating membrane rigidization both in hydrocarbon core region and near-surface bilayer region.

It seems likely that fluctuations of the C:PL ratio, followed in both the options of our experiment, result most probably from membrane phospholipid degradation and essentially not from membrane cholesterol accretion. Considering this, it should be emphasized that the total amount of membrane lipid decreases. Hence, assuming the lipid bilayer com-

pressibility to be maintained in certain intimate limits, one might expect the progressive degradation of total bulk of membrane lipids to impose rather restricted fluctuations of membrane lipid fluidity, as evaluated by fluorescence anisotropy values. There is accumulating experimental data suggesting that alloxan should be considered as a causative factor in the enhanced sensitivity of red cells to peroxidation [2, 3]. The evidence for lipid peroxidation-induced membrane rigidization is nowadays overwhelming [19–22].

Alloxan can be reduced *in vitro* to dialuric acid, which auto-oxidizes spontaneously forming H_2O_2, O_2^- and $HO^\cdot$, and the detection of hydrogen peroxide in erythrocytes after treatment with alloxan implies similar conversion of alloxan to dialuric acid *in vivo* [2]. Such aerobic reduction and oxidation cycling reactions are sources of reactive oxygen species, which induce highly localized tissue damage [1, 2]. The specificity of damage appears to depend upon a selective accumulation of alloxan-dialuric acid redox couple in pancreatic beta cells. Other tissues and organs appeared essentially unaffected by diabetogenic doses of alloxan, and the only exception was observed in vitamin E deficiency, where dialuric acid induced erythrocyte hemolysis [3].

Whether reported herein alloxan-induced lowering of membrane fluidity results mainly from altered membrane lipid status remains to be established, however, the bulk of convincing evidence concomitantly encourage the speculation upon the fact that the main functional events of alloxan action are located at the plasma membranes [1–3].

Erythrocytes might be expected to be highly susceptible to peroxidation as they contain a powerful transition metal catalyst and their membranes are rich in polyunsaturated fatty acids [23].

Beyond its relevance to the mode of action of alloxan, the experimental data presented herein also suggest that the mechanism of alloxan action certainly contributes to the essential alterations in cell membrane dynamics.

The relevance of changes induced by alloxan in the membranes of intact erythrocytes and isolated ghosts preparations is briefly evidenced.

Haem components, many of which are intimately associated with the phospholipids of the biological membranes, were shown to be the best promoters in lipid peroxidation both *in vitro* and *in vivo*. Hence, the oxidative interaction of haemoglobin with membrane lipid constituents is of great importance, as it can be regarded as a key step in membrane lipid peroxidation and hemolytic process [23].

Though a differential extension of found alterations might be expected, due to haem catalytic peroxidation effect [24], they are not really much more profoundly expressed in the experiments with intact erythrocytes. The only, if noteworthy, nonconcomitant tendencies are fluctuations of membrane cholesterol content (which diminishes evidently more rapidly in isolated erythrocyte membranes) might be due to possible releasing of microvesicles of different lipid composition (i.e. enriched with cholesterol) from the ghosts incubated with higher alloxan concentrations.

In both the cases the range of damage in alloxan-treated cell membranes turned out to depend strongly upon the intensity of peroxidation process, given the performed alloxan concentrations. Time dependence of changes accumulation might also be expected to occur, and it is really the case.

Our findings are in keeping with numerous reports, where induced membrane lipid peroxidation implied the decrease of membrane phospholipid : cholesterol ratio and increase of DPH or ANS anisotropy [19–21].

Conflicting results as regards to the effect of alloxan on isolated erythrocyte ghosts were obtained recently by one of us (Jóźwiak, unpublished data). In the study mentioned, alloxan was found to induce a significant increase of membrane fluidity at the depth of C-12, as evidenced by ESR spectra of doxylstearic acid derivatives, and was out of any influence at the depth of C-16. That report, when considered with regard to our present findings, seems surprising. However, these two studies need not be mutually exclusive, since the methodological approach carried out in each case, may explain why membrane fluidity fluctuations found are oppositely directed. Alloxan, which is believed to destroy double bonds in polyunsaturated fatty acids of membrane phospholipids via free radical mechanism, favours the gap creation in phospholipid matrix of lipid bilayer, mainly at the depth range of C-7 to C-12, where the bilayer is expected to contain a large proportion of fatty acid double bonds susceptible for peroxidation. Hence, the order parameter and freedom of rotational diffusion decreases in a differential manner, which is dependent upon the ESR probe location. The rotational motion may be thus greater or slower subject to the fitness of a probe location and gap occurrence. A fluorescent probe, like DPH or ANS, reflects the resultant microviscosity of lipid bilayer, respectively, at the deeper region of hydrophobic core or near bilayer surface, and as such can monitor lipid mobility alterations affected by C : PL ratio or even crosslinking of membrane components conditioning acyl chain flexibility [22]. However, it should be realized that fluctuations of the amounts of TBARS and followingly the correlation of these changes with relevant alterations of anisotropy values, reported herein, may comply an additional interference effect due to natural membrane sugars and/or proteins elevating the determined TBARS levels.

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