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THE EFFECT OF *O*-(β-HYDROXYETHYL) RUTOSIDE ON PLATELET INTERMEDIARY METABOLISM IN NORMAL AND STREPTOZOTOCIN DIABETIC RATS

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Abstract—On the basis of measurements of platelet glycolytic intermediates from glycogen to pyruvate, washed platelet suspensions from rats with diabetes induced 3–7 months previously with streptozotocin showed a 60 per cent reduction in fructose-6-phosphate and 200 per cent increases in fructose-1,6-diphosphate, 3-phosphoglycerate and pyruvate when compared to normal. These results coupled with increases in the products of glycolysis during aggregation induced by 5μM ADP, suggested increased glycolytic activity in diabetic rat platelets. *O*-(β-hydroxyethyl)-rutosides (50 mg s.c./100 g for 3 days) reduced glycolytic intermediates in platelets from both normal and diabetic rats to about the same level. ATP and glycogen levels were also reduced. An increase in platelet glycolytic activity may be connected with increased platelet adhesiveness and ease of ADP-induced aggregation reported in humans with deteriorating diabetic microangiopathy.

FOLLOWING reports of increased platelet adhesiveness in diabetes mellitus,[1–5] it was found that platelets from diabetic humans with a deteriorating retinopathy were more sensitive than those of other diabetics or non-diabetics to the aggregating action of ADP *in vitro*,[6] and the aggregates formed dispersed more slowly. An increased clumping of platelets, should it occur *in vivo*, might contribute to the development of diabetic microangiopathy by the obstruction of local blood vessels; with ischaemic damage occurring to the capillary wall.

One of the metabolic changes which occur during platelet aggregation is a stimulation of glycolysis[7,8] and inhibition of platelet intermediary metabolism is accompanied by a reduction in adhesion and aggregation.[9] Thus an agent which suppresses platelet glycolysis, without having side effects which would exclude its use, might be useful for the prevention of microembolisation.

Hydroxyethyl substituted rutosides have been shown to diminish the severity of diabetic retinopathy observed by retinal fluoresceinography[10] and we have shown that they retard the development and reduce the extent of a retinopathy induced in rats by β,β′-iminodipropionitrile.[11] We, therefore, investigated the effect of their *in vivo* administration to rats on the glycolytic intermediates of washed platelets before and during ADP-induced aggregation *in vitro*. Experiments were carried out on normal rats and on rats 5–7 months after being made diabetic with streptozotocin, since this form of chronic experimental diabetes is one of the few in which changes similar to the typical retinopathy of diabetes mellitus have been seen.[12]

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MATERIALS AND METHODS

Animals and treatment. Male Wistar rats (150–200 g) were maintained on M.R.C. diet 41B and water *ad lib.* Animals were made diabetic by the injection into the tail vein of 50 mg streptozotocin/kg. The dose was dissolved in 0·2 ml of a citrate–phosphate buffer pH 4·5 (12·9 mg citric acid, 27·6 mg $Na_2HPO_4.12H_2O$/ml) immediately before injection. Diabetic rats showed a positive test for urine glucose (Clinistix) after 3 days and plasma glucose was measured fluorimetrically as described below when the animals were killed 5–7 months later.

Two groups of 8–10 normal and diabetic rats acted as controls and two further groups were injected s.c. with 50 mg *O*-(β-hydroxyethyl)-rutoside (HR), in 0·25 ml H_2O/100 g/day for 3 days. Blood was extravasated 1–2 hr after the last injection.

Platelet preparation. Rats were anaesthetised with Nembutal (5 mg i.p./100 g) and blood was obtained by cannulation of the abdominal aorta with a 1·02 or 1·34 O.D. Portex nylon cannula previously rinsed with the acid citrate dextrose (ACD) solution of Aster and Jandl.[13] Blood was drawn into a 10 ml plastic disposable syringe, diluting with ACD from a 2 ml plastic syringe to give a final ACD concentration of 1 in 6 using a three way nylon stopcock. Platelet-rich plasma was prepared by centrifuging the citrated blood in a siliconised tube at 170 *g* for 20 min. Washed platelets were prepared at 37° using the method of Mustard *et al.*[14] omitting calcium throughout. The apyrase concentration was 5 mg/100 ml and the final suspending fluid was their solution A. The pH of these media was monitored, since it was found that it could rise 1·5 units due to the loss of CO_2 unless kept carefully covered. With an increase in pH the platelets, especially from diabetic animals, tended to aggregate spontaneously and could not be resuspended. The platelet count was determined in the final suspension by the method of Brecher and Cronkite[15] and the suspension diluted to give 7–8 × 10^5 platelets/μl.

Platelet aggregation. This was carried out on 1·0 ml samples of platelet suspension according to Heath *et al.*,[6] using an EEL aggregation meter and a Bryans recorder (Model 27,000). Aggregation was initiated by the addition of 10 μl of the following solutions: $CaCl_2.2H_2O$, 25·5 mg/ml, Fibrinogen, 50 mg/ml and $ADP.Na_3$, 0·5 mg/ml. Acid extracts of the platelets were obtained (1) before aggregation, that is before the addition of $CaCl_2$, fibrinogen and ADP, (2) at the point of maximum aggregation as followed in the aggregation meter (2 5 min after ADP addition) and (3) at the point of maximum disaggregation (10–15 min), by the addition of 50 μl 10N $HClO_4$. After 20 min at 4° the extracts were transferred to 5 ml conical centrifuge tubes and spun at 4000 *g* for 10 min. 0·95 ml extract was neutralised with approximately 40 μl 5 M K_2CO_3 and the samples stored, suspended in acetone–dry ice (−79°), until assayed.

Assay of intermediates. Modifications of several fluorimetric methods[16–18] were used for the determination of the following glycolytic intermediates, glucose-1-phosphate (GIP), glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-diphosphate (FDP), dihydroxyacetone phosphate (DAP), glyceraldehyde-3-phosphate (GAP), 3-phospho- and 2-phospho-glycerate (3PG and 2PG), phosphoenolpyruvate (PEP), pyruvate (PYR), and ATP using an EEL fluorimeter Model 244 and Bryans recorder (Model 27000). 0·2 ml platelet extract was added to 2·0 ml of the appropriate buffer for each series of measurements as previously described.[19] PYR, PEP, 2PG and 3PG were measured in the 20 mM imidazole buffer used for FDP

rather than in phosphate buffer due to the formation of calcium phosphate precipitates derived from the calcium added to initiate platelet aggregation. The contents of platelet intermediates were expressed as nmoles/10^{10} platelets. Plasma glucose was also measured fluorimetrically after appropriate dilution.[16]

Platelet glycogen was determined in platelets from four groups of rats with the same treatment as described above, both before and after the washing procedure described in platelet preparation. KOH extracts were made of platelets centrifuged from platelet rich plasma or washing medium at 4000 *g* for 10 min and glycogen assayed by the anthrone method.[20]

Results were analysed statistically and significant differences between the means (calculated as P values) are indicated when $P \leqslant 0{\cdot}05$.

Materials. Streptozotocin was a gift from Upjohn, Kalamazoo, Michigan, U.S.A. and O-(β-hydroxyethyl)-rutoside was a gift from Zyma S.A. Nyon, Switzerland. Apyrase, Fibrinogen (Bovine Type III), Glycogen (rabbit liver Type III) and Imidazole (low fluorescence grade III) were obtained from Sigma, London and all enzymes, substrates and cofactors from Boehringer, London.

RESULTS

Platelets from diabetic rats tended to aggregate spontaneously usually during centrifuging after the first resuspension in the medium of Mustard *et al.*[14] Six out of ten samples were lost in this way from the diabetic group whereas it did not occur with normal rat platelets unless the pH of the medium was high as described above.

Nonaggregated platelets. From Table 1 it can be seen that diabetes had no effect on the ATP content of washed rat platelets. Three days HR treatment however, led to a significant reduction in ATP to 80 per cent of normal in normal rat platelets and to 89 per cent of diabetic in diabetic rat platelets. Looking at the overall pattern of intermediates in Fig. 1 for non-aggregated platelets, diabetes resulted in significant increases in fructose-1,6-diphosphate (FDP), 3-phosphoglycerate (3PG) and pyruvate (PYR) to about 200 per cent of normal. There was a decrease in fructose-6-phosphate to 60 per cent of normal which was also significant. Glucose-1-phosphate (GlP) and glucose-6-phosphate were also raised. HR treatment resulted in a similar pattern in both normal and diabetic rat platelets. In both there were significant reductions to between 20–60 per cent of normal in the intermediates between and including FDP to PEP.

Aggregated platelets. ATP levels were the same at the point of maximum aggregation as in non-aggregated platelets (Table 1) and HR treatment caused a similar

TABLE 1. EFFECT OF HR TREATMENT ON ATP CONTENT OF NORMAL AND DIABETIC RAT PLATELETS

Number of animals	Normal 9	Normal + HR 7	Diabetic 4	Diabetic + HR 3
Non-aggregated	486·2 ± 49·6	389·6 ± 23·6*	513·2 ± 22·7	461·2 ± 12·2*
Aggregated	525·6 ± 68·7	394·9 ± 24·5*	534·2 ± 48·1	460·6 ± 10·8*
Disaggregated	526·7 ± 59·3	402·4 ± 15·7*	447·9 ± 69·6	507·0 ± 18·2*

HR was injected s.c 50 mg/100 g/day for 3 days. Platelets were caused to aggregate by the addition of $CaCl_2.2H_2O$, 0·255 mg, fibrinogen, 0·5 mg and ADP, 0·05 mg/ml platelet suspension. Results are expressed in nmoles/10^{10} platelets as mean ± S.E.

* Indicates significant effect of HR on normal or diabetic control, P = 0·05 or less.

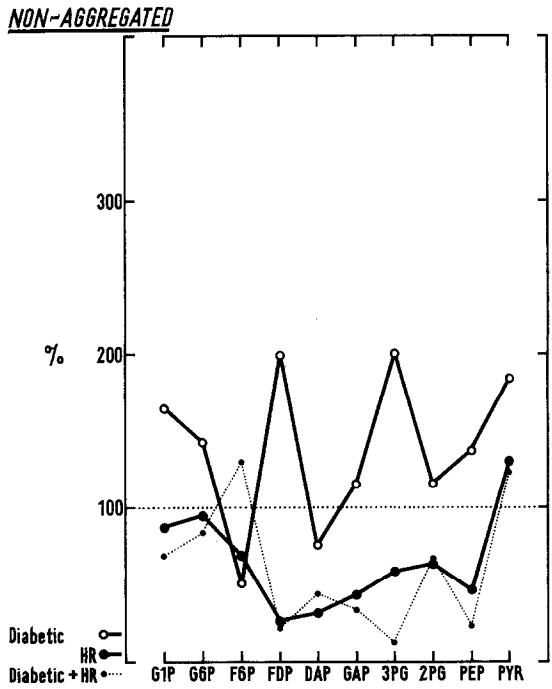

FIG. 1. Glycolytic intermediates in washed rat platelets before the addition of aggregating agents. Levels in platelets from diabetic rats (n = 4), diabetic rats treated with HR (50 mg/100 g, s.c.) (n = 3) and HR treated normal rats (n = 7) compared as a percentage of those in normal rat platelets (100 per cent) (n = 9). These normal levels in metabolic sequence from G1P to PYR, expressed as nmoles/10^{10} platelets ± S.E., were 3·60 ± 0·88, 11·13 ± 2·03, 5·81 ± 0·88, 21·22 ± 6·37, 32·17 ± 3·22, 12·42 ± 2·85, 21·92 ± 3·96, 10·43 ± 2·83, 19·44 ± 4·59 and 560·2 ± 128·7. Abbreviations are explained in the Methods section.

reduction. The levels of intermediates from G1P to FDP in diabetic rat platelets were greater than normal, whereas the remainder excepting PEP and PYR were decreased. This was only a trend since G1P and 2PG were the only intermediates in which the change was significant, G1P being increased from 4·29 ± 0·85 (S.E.) to 7·46 ± 0·75 nmoles/10^{10} platelets and 2PG being decreased from 5·62 ± 1·63 to 1·12–0·04 nmoles. HR treatment of diabetic rats resulted in an increase in F6P from 4·85 ± 0·90 to 9·23 ± 0·95 nmoles (190 per cent), and a decrease in FDP (5·80 ± 0·63) to 40 per cent of the level in normal aggregated platelets (14·86 ± 2·29). Although there were reductions in all intermediates except pyruvate in platelets from HR treated normal rats, the only significant reductions were in FDP from 14·86 ± 2·29 to 7·73 ± 0·92, DAP from 38·55 ± 8·55 to 13·49 ± 1·79, GAP from 13·76 ± 1·67 to 9·87 ± 0·68 and PEP from 19·42 ± 4·65 to 9·31 ± 1·95 nmoles/10^{10} platelets.

Disaggregated platelets. The ATP content of normal platelets was unaltered at the point of maximum disaggregation compared to the level before aggregation, with and without HR treatment (Table 1). In the diabetic rat platelets the ATP content was reduced at this point but not significantly. A rise in ATP content after HR treatment reversed the reduction which otherwise occurred in these experiments with this drug. G1P to F6P levels were unaltered by diabetes or HR treatment, apart from a tendency for them to be higher in the former and the same or lower in the latter com-

pared to normal. FDF to GAP levels were decreased in all three groups to between 40–60 per cent of levels in normal disaggregated platelets. In diabetic rats there were large increases compared to normal in 3PG from 20·61 ± 6·93 to 44·07 ± 8·13, 2PG from 6·13 ± 3·07 to 24·26 ± 8·91, PEP from 15·11 ± 3·68 to 24·80 ± 4·21 and PYR from 482·4 ± 11·7 to 998·3 ± 15·8 nmoles/10^{10} platelets. HR treatment of diabetic rats reduced all these intermediates except PYR to 28 per cent of normal in the case of 3PG, 5·77 ± 0·93, 43 per cent for 2PG, 2·64 ± 0·82, and 10 per cent for PEP, 11·48 ± 0·29. These intermediates were also reduced by treating normal rats with HR, but to a less extent.

Platelet glycogen. In a separate series of rats HR treatment had no effect on platelet glycogen levels before or after washing, Table 2. However, washing caused a significant reduction in glycogen level to about one half of that of platelets centrifuged from platelet-rich plasma. The glycogen content of unwashed diabetic rat platelets was 85 per cent of that from normal platelets and unaffected by washing. HR treatment reduced washed platelet glycogen from diabetic rats to 65 per cent of the content of washed diabetic rat platelets.

TABLE 2. EFFECT OF HR TREATMENT ON GLYCOGEN LEVELS IN NORMAL AND DIABETIC RAT PLATELETS BEFORE AND AFTER WASHING

	No.	Untreated	No.	HR treated
Normal				
Unwashed	4	348·0 ± 11·3	4	321·7 ± 9·7
Washed	4	189·5 ± 7·8*	4	186·2 ± 6·4*
Diabetic				
Unwashed	4	296·2 ± 8·4†	—	—
Washed	4	296·5 ± 14·3	3	194·0 ± 10·5

Platelets were washed twice with the medium of Mustard *et al.*[14] HR was injected s.c. 50 mg/100 g/day for 3 days. Results are expressed in μg/10^{10} platelets as mean ± S.E.

* Indicates levels which differ significantly from unwashed.

† Diabetic level significantly different from normal, P = 0·05 or less.

DISCUSSION

The ocular lesions described by Leuenberger *et al.*[12] in rats 6 months after being made diabetic with streptozotocin included retinal microangiopathic changes which are seen in humans with diabetes mellitus but rarely seen in experimentally induced diabetes. In this study we found that the platelets of these chronically diabetic rats tended to aggregate spontaneously under certain conditions unlike those from normal animals. These altered platelets could contribute to the retinopathy seen in the rat,[21] especially since increased platelet adhesiveness[1–5] and enhancement of aggregation[6] may be implicated in human diabetic microangiopathy. The development of increased platelet function could be due to a factor, in the plasma or in the platelets, affecting the platelet cell membrane or the metabolic processes associated with aggregation, one of which is glycolysis.[7,8]

We have found that before aggregation platelets from chronically diabetic rats contained smaller amounts of fructose-6-phosphate and increased amounts of fructose-1,6-diphosphate, 3-phosphoglycerate and pyruvate. These intermediates are

either substrates or products of reactions catalysed by phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase. This pattern of intermediates in diabetic rat platelets indicates increased enzyme activity, either allosterically or by increased synthesis, at these well known glycolytic control points.[16,18,22] The smaller increases in GlP and G6P further confirm an increased glycolytic activity. This increased activity, if it also occurred *in vivo*, was probably mediated by an increased glucose uptake from the plasma, not necessarily related to the high plasma glucose (>300 mg/100 ml) of the diabetic rats, since it was seen in washed platelets suspended in a medium containing the same amount of glucose as the normal platelets. If the K_m (D-glucose) for rat platelet hexokinase is in the same order as that for calf brain,[23] $8{\cdot}0 \times 10^{-6}$ M, it would be saturated at the normal blood glucose level anyway. Detwiler[24] has suggested that the level of hexokinase in normal rat platelets did not limit glycolysis and that platelets are insulin insensitive. This tends to confirm an increased hexokinase activity in diabetic rat platelets induced allosterically or by increased synthesis.

HR treatment for 3 days reduced the content of glycolytic intermediates of normal rat platelets before aggregation. Those of diabetic rat platelets were reduced to very similar values and in this case there was a reduced phosphofructokinase activity since F6P was increased and FDP decreased (Fig. 1), which was also seen in diabetic rat platelets at the point of maximum aggregation (see Results section). Both glycogen and ATP levels were reduced as well in diabetic rat platelets, but only ATP was reduced in normal rat platelets. The changes in ATP content may have been in either the metabolically active or inactive pools said to occur in platelets by Holmsen.[25] These results show that HR probably causes a reduction in platelet intermediary metabolism in both normal and diabetic rats and that the effect in diabetic rats is greater, reducing both glycogen and intermediate levels to near those found in normal HR treated rat platelets. It has recently been demonstrated[26] that HR *in vitro* inhibits the uptake of adenosine by human platelets. If HR inhibits the uptake of adenosine by rat platelets *in vivo*, this might explain the lower levels of ATP observed.

Glycogen levels dropped during the washing process in normal rat platelets but not in those from diabetic rats. This fall may be related to the loss of other intraplatelet material which occurs during normal careful manipulation[27] and of glycolytic intermediates during thrombin and epinephrine induced aggregation.[28] These changes serve to emphasise the caution with which any data from isolated platelets should be interpreted. However, the results shown for platelets before aggregation are likely to be representative of the intracellular state of affairs since extracts were made soon after the final resuspension. The levels of intermediates at the point of maximum aggregation and in disaggregated platelets, however, are without doubt those within and outside the platelets. They cannot be compared with Detwiler's results,[28] showing levels of glycolytic intermediates in the first few seconds after causing platelet aggregation with thrombin or epinephrine, since he attempted an allowance for intermediates released and because the time scale was different. The results for the aggregated platelets, however, do show a possible increase in glycolysis in diabetic rat platelets. HR reduced this in diabetic rats and also caused an inhibition of phosphofructokinase as discussed above. At the point of maximum disaggregation, in the diabetic rat platelet suspensions, there was a large accumulation of intermediates at the

terminal end of glycolysis which could also indicate a higher glycolytic rate than normal. This was entirely cancelled by HR treatment, giving a pattern very similar to HR treated normal platelets, which was also seen in platelets before aggregation.

In summary we have shown that HR reduces the increased glycolytic activity which probably occurs in platelets from rats made chronically diabetic with streptozotocin. Whether an increased activity is associated with increased platelet adhesiveness, which is said by some[1–5] to occur in diabetes and others[6,29] not, it cannot be said. It may be related to the increased ability of ADP to cause aggregation of platelets from patients with a deteriorating retinopathy[6] however, and the effect of HR on platelet glycolysis might be implicated in the prevention of experimental thrombosis[30] and reduction in the severity of diabetic retinopathy.[10]

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