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STUDIES ON THE EFFECTS OF ββ′-IMINODIPROPIONITRILE AND O-(β-HYDROXYETHYL)-RUTOSIDE ON ADP-ACTIVATED AGGREGATION OF RAT PLATELETS IN RELATION TO THE DEVELOPMENT OF DIABETIC MICROANGIOPATHY

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Abstract *In vitro* platelet aggregation in response to ADP has been shown to be increased in diabetics, especially those with deteriorating retinopathy. The administration to animals of ββ′-iminodipropionitrile (IDPN), a lathyrogen, induces a retinopathy very similar to that of the diabetic and therefore serves as a useful model for this condition. The *in vitro* and *in vivo* effects of IDPN and O-(β-hydroxyethyl)-rutoside (HR), a compound which retards the development of IDPN-retinopathy, on the ADP-activated aggregation of platelets from rats were assessed. The continuous administration of IDPN and HR, and a combination of both, to rats showed that HR brought back to normal the increased ADP-activated aggregation caused by the administration of IDPN. Neither IDPN nor HR affected the ADP-aggregation of rat platelets *in vitro*. The implications of these findings and their relevance to an animal model for diabetic retinopathy are discussed.

The administration of ββ′-iminodipropionitrile (IDPN), either orally or subcutaneously, to rats causes the development of a retinal microangiopathy in which the pathological changes are similar in many respects to those observed in human diabetic retinopathy [1–3]. It has also been shown that treatment of rats with hydroxyethylrutosides (HR) retards the development of the IDPN-induced retinopathy [4]. Recently, it has been demonstrated that the platelets from human diabetic subjects with deteriorating retinopathy are more sensitive to the aggregating action of adenosine diphosphate (ADP) and that the aggregates formed do not disaggregate again so readily [5]. It has been suggested that diabetic microangiopathy may be initiated by episodic capillary occlusions caused by the intravascular aggregation of hypersensitive platelets during periods of poor metabolic control [6].

The mechanism by which IDPN initiates microvascular changes in the rat is not known. The possibility exists that following the treatment of animals with IDPN the platelets would become hypersensitive and aggregate, the microthrombi so formed occluding the capillaries and the resulting localized anoxic areas setting in train the subsequent pathological changes. It was decided, therefore, to study the effects of the *in vivo* administration of IDPN and HR on the aggregating action of ADP *in vitro* on the platelets from these treated animals.

MATERIALS AND METHODS

Chemicals. ββ′-Iminodipropionitrile (IDPN) was obtained from Kodak Ltd., Kirkby, Liverpool, converted into the hydrochloride and recrystallized from 95% v/v ethanol. O-(β-hydroxyethyl)-rutoside was generously supplied by Zyma (S.A.), Nyon, Switzerland. Trisodium adenosine diphosphate (ADP) was obtained from Boehringer Corporation (London) Ltd. A 1·1 mM solution in 0·9% w/v NaCl was prepared and stored in 1-ml portions at −10°. 22 μM ADP solutions were prepared freshly each day from these stock solutions.

Animals. Male Wistar rats (200–250 g) were maintained on MRC diet 41 and water *ad lib.*

IDPN was administered in the drinking water at a concentration of 2 mg/ml for 7 days. These animals were left for a further 8 days before the platelets were harvested.

HR was administered in the drinking water for 21 days at a concentration of 1·66 mg/ml. The platelets from these animals were harvested on the 22nd day.

When treatment with IDPN (2 mg/ml) and HR (1·66 mg/ml) was combined HR was administered for 21 days, IDPN was given on days 8–14 and the platelets were harvested on the 22nd day. It was assumed that the rats, on average, drank 15 ml of water per day and so the approximate daily dose of HR was 25 mg and of IDPN was 30 mg.

Blood specimen. Rats were anaesthetized by the intraperitoneal injection of Nembutal (5 mg/100 g body wt). Blood was collected by cannulation of the abdominal aorta. The aorta was carefully separated from the vena cava and two clamps, 1 cm apart, were placed on the abdominal aorta proximal to the bifurcation. A small longitudinal incision was made close to the lower clamp and this was expanded with fine forceps to allow the insertion of the plastic cannula as far as the upper clamp. This was released and the cannula was eased a further 2–3 cm so that the blood collected did not come in contact with any damaged cells. The blood was collected through the cannula (o.d. 1·34 mm) attached to a three-way plastic tap which connected to a 1·0 ml plastic syringe containing 3·8% w/v sodium citrate solution and to an empty 10 ml plastic syringe. Before use, the cannula, the 10 ml syringe and the three-way tap were rinsed with 3·8% w/v sodium citrate solution. As blood was withdrawn into the 10 ml syringe, sodium citrate solution was allowed to enter so that the final volume of citrated blood was ten times the volume of sodium citrate added.

Platelet-rich plasma (PRP). This was prepared by centrifuging the citrated blood in a siliconized glass tube at 170 *g* for 20 min. The supernatant PRP was transferred, using a polythene Pasteur pipette, to a plastic container. PRP from two rats was combined. The platelet count was determined by the method of Brecher and Cronkite [7] using phase contrast microscopy on a 1:2000 dilution of the PRP with platelet diluting fluid (Clintech Ltd). A suitable portion of PRP was centrifuged at 1200 *g* for 15 min. The supernatant platelet poor plasma (PPP) was decanted and used to dilute the PRP so that it contained 300,000 platelets/mm^3. The remaining blood cells and plasma were centrifuged at 1200 *g* for 15 min to obtain PPP which was used as a blank solution in the aggregation meter.

Platelet aggregation. This was determined by a modification of the method of Born [8] using a microaggregation meter designed by Mr. P. R. E. Wallace of the Nuffield Institute of Comparative Medicine. The voltage changes were recorded on a Bryans recorder (Model 27,000). All determinations were made on samples stirred at a constant rate by a siliconized wire over an external rotating magnet and they were recorded at a chart speed of 3 cm/min. 0·2 ml PPP was transferred to a siliconized glass tube (5 × 50 mm) and placed in the aggregation meter. The instrument was adjusted for all samples of PPP to give the same voltage output. Then 0·2 ml PRP which contained 300,000 platelets/mm^3 was incubated for 5 min at 37°. It was placed in the aggregation meter and the voltage change recorded. 0·02 ml 22 μM ADP was added to give a final concentration of 2 μM and the changes in voltage recorded until aggregation and disaggregation were complete.

Experiments to test the effect of HR *in vitro* were carried out on PRP obtained from pairs of untreated rats. The PRP was adjusted to 300,000 platelets/mm^3 and PPP was obtained as already described. In order to overcome the change in colour caused by the addition of the HR solution to the PRP, 0·02 ml 5·5 mM HR in 0·9% w/v NaCl was added to 0·2 ml PPP and the instrument adjusted to give the same voltage as that used in the *in vivo* experiments. 0·02 ml 5·5 mM HR in 0·9% w/v NaCl was added to 0·2 ml PRP to give a final concentration of 0·5 mM HR and this was incubated at 37° for 5 min. 0·02 ml 24 μM ADP was added to give a final concentration of 2 μM and the changes in voltage were recorded during aggregation and disaggregation of the platelets. A control experiment was carried out by substituting 0·02 ml 0·9% w/v NaCl for the HR solution after each determination of the effect of HR so that the possible alterations due to the ageing of the platelets would be eliminated. The *in vitro* effect of 5·5 mM IDPN on the aggregating action of 2 μM ADP was tested in the same way.

RESULTS

When low concentrations of ADP were added to a stirred suspension of platelets at 37° the following sequence of events took place in the translucency of the suspension. Firstly, owing to the diluting effect of the ADP solution, an increase in light transmission occurred. This was immediately followed by a decrease in transmission caused by the platelets becoming spherical. The aggregation of the platelets resulted in a rapid increase in light transmission which reached a maximum after approximately 60 sec in the presence of 2 μM ADP. The platelet aggregates so formed were not stable and a decrease in light transmission occurred reflecting the extent of disaggregation. The maximum aggregation attained and the rates of aggre-

Table 1. Effects of the *in vivo* administration of HR (25 mg/day) for 21 days on the aggregating action of 2 μm ADP on platelets from normal and IDPN-treated rats

Treatment	Determinations	Maximum aggregation (%)	Maximum aggregation rate (%/sec)	Disaggregation rate (%/sec)
None	23 (6)	35·1 ± 1·9	0·84 ± 0·04	0·55 ± 0·03
HR	20 (8)	26·6 ± 1·3	0·77 ± 0·04	0·41 ± 0·02
IDPN	24 (6)	46·9 ± 0·9	1·36 ± 0·06	0·86 ± 0·07
HR + IDPN	32 (8)	30·4 ± 0·9	0·87 ± 0·02	0·48 ± 0·02

The number of rats are indicated in parentheses.

Table 2. Effects of incubating rat platelets with either 0·5 mM HR or 0·5 mM IDPN on the aggregating action of 2 μM ADP

PRP	Determinations	Maximum aggregation (%)	Maximum aggregation rate (%/sec)	Disaggregation rate (%/sec)
Controls	37	48·2 ± 1·43	1·15 ± 0·06	0·63 ± 0·03
+HR	37	43·9 ± 2·35	1·03 ± 0·05	0·61 ± 0·03
Controls	24	38·2 ± 1·33	0·91 ± 0·04	0·81 ± 0·03
+IDPN	24	39·2 ± 1·16	0·96 ± 0·04	0·85 ± 0·03

gation and disaggregation were calculated from the recorded tracings after the addition of ADP.

The maximum increase in light transmission represents the maximum degree of aggregation. It is expressed as a percentage of the difference in light transmission between PPP and PRP and this is taken as 100 per cent [5]. Maximum aggregation rate is taken as the maximum slope while aggregation is occurring. The disaggregation rate is the maximum slope during disaggregation and each is expressed as the percentage change in light transmission per second.

Studies in vivo. The effects of the *in vivo* administration of HR (25 mg/day) for 21 days to normal rats on the aggregating action of 2 μm ADP are shown in Table 1. This treatment with HR caused a significant reduction ($P < 0·01$) in all three parameters of aggregation when compared with those for normal rats.

When rats were treated with IDPN (30 mg/day) for 7 days there were significant increases ($P < 0·01$) in maximum aggregation and in the rates of aggregation and disaggregation initiated by the action of 2 μM ADP on PRP obtained from these rats 8 days after the termination of IDPN administration (Table 1).

In order to investigate the effect of HR on IDPN-treated animals, rats were pre-treated with HR (25 mg/day) for 7 days. This level was maintained during the next 7 days in which IDPN (30 mg/day) was administered and for a further 7 days. Table 1 shows that the sensitivity of the platelets to 2 μM ADP was significantly reduced ($P < 0·01$).

Studies in vitro. The effects of 0·5 mM HR or 0·5 mM IDPN on rat platelet aggregation induced by 2 μM ADP are shown in Table 2. It will be seen that the treatment of rat platelets *in vitro* with either HR or IDPN caused slight changes in all three parameters but none of these changes was statistically significant.

DISCUSSION

The administration of IDPN, 25 mg/day for 7 days, has been shown to cause a mild retinopathy [4] in rats and treatment of these animals with HR, 25 mg/day for 21 days, greatly ameliorates this condition. It has also been shown that during the treatment of rats with IDPN there is an increase in the soluble collagen content of the aorta [9] and that the level of soluble collagen is restored to normal after treating with HR. In this case experiments were conducted for a period of 33 days in which the flavonoid was administered for the whole of that time and the lathyrogen from the 6th to the 13th day.

The mechanism by which IDPN induces the pathological changes in the retinal microvasculature is not known but it is possible that occlusion of the capillaries by microthrombi of platelet aggregates might occur; the platelets having been sensitized by the IDPN-liberated soluble collagen.

It was not known whether the ADP-sensitive platelets in the retinopathic rats were responsible for the development of the retinopathy or were a secondary phenomenon. Soluble collagen, especially lathyric collagen, has been shown to be one of the most potent platelet aggregating agents [10] and it was possible that platelets from IDPN-treated animals had in some way been sensitized by exposure to abnormal collagen *in vivo*. In order to investigate this possible effect of IDPN and HR on rat platelet behaviour towards ADP it was decided to study the action of these substances *in vitro*. It was found that neither of these substances significantly affected the aggregating action of ADP on rat platelets when added directly to the incubating media. Thus it would appear that HR protects the experimental animals against the platelet sensitizing action of the lathyrogen, IDPN, only *in vivo*. Other investigators have shown that HR *in vivo* reduces capillary permeability and the charge on the capillary endothelium [11]. These results would seem to indicate that HR has some effect on the endothelium. Entrican *et al.* [12] have also shown that HR reduces or prevents the inflammatory response to bradykinin in *in vivo* hamster cheek pouch preparations.

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