

Neutral Red Changes Arginine-Induced Glucagon and Insulin Response in the Rat

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Abstract: Rats were treated with neutral red (amino-dimethyl-amino-toluanizine hydrochloride, NR) twice weekly, 5 ml/kg b.wt.⁻¹ of a 2% solution, for five weeks in order to investigate damage to pancreatic A-cells. After 1-5 weeks of treatment plasma glucagon levels were increased to about two times control values. Plasma glucose was maintained at control values. Following arginine stimulation, 8 µg intravenously 100 g b.wt.⁻¹ min.⁻¹ for 30 min., plasma glucagon and insulin concentrations increased 10-fold in normal rats. After NR treatment for 1 and 2 weeks the arginine induced glucagon response was reduced to a two-fold increase, after 5 weeks NR treatment arginine again increased glucagon concentrations 10-fold. There was no change in the 10-fold increase of insulin concentrations from arginine infusion during the NR treatment. Immunohistochemical examination of pancreas demonstrated unchanged number and size of Langerhans' islets. The insulin immunoreactivity of the B-cells appeared constant 0-5 weeks during NR treatment, while glucagon immunoreactivity appeared to lower in the A-cells from animals treated for one or two weeks with NR. After three to five weeks of NR treatment, pancreatic glucagon immunoreactivity appeared restored. We conclude that NR leads to a release of glucagon from pancreatic A-cells, with subsequent hyperglucagonaemia, maintained during repeated administrations. Such repeated administrations lead to a transient »glucagon insufficiency« on top of the hyperglucagonaemia, consisting of restricted ability to further release of glucagon, presumably because of pancreatic A-cell glucagon depletion.

It is experimentally possible to destroy pancreatic B-cells with alloxan or streptozotocin, but there is no method to produce damage to pancreatic A-cells. About 20 years ago it was reported that acute administration of the indicator dye Neutral Red (NR) produced hyperglycaemia and pancreatic-A cell destruction (Okuda & Grollman 1966). Soon after it was demonstrated that serum glucagon was increased concomitantly with decreased pancreatic content (Nevis *et al.* 1968). Okuda & Grollman (1966) also reported that prolonged administration of NR caused severe hypoglycaemia presumably by destruction of the pancreatic A-cells. Later studies on hypophysectomized rats suggested that the hyperglycaemia was mediated through an adrenal catecholamine release (Loubatieres *et al.* 1974). The destruction of pancreatic B-cells by chronic NR administration, however, could not be confirmed (Loubatieres *et al.* 1974; Federspil *et al.* 1972).

In special situations glucagon is released with subsequent high plasma concentrations, e.g. during hepatic regeneration. We have earlier reported that exogenous glucagon administration for a 3 week period leads to reduced ability of alanine to stimulate release of glucagon (Petersen *et al.* 1987a), and that under such conditions the normal increase in glucagon plasma concentration is hampered and hepatic regeneration is stimulated (Petersen *et al.* 1987b). It appeared possible that NR could produce a similar state with glucagon deficiency. In the present study we have examined the response to the potent glucagon stimulator arginine in NR treated rats. We

investigated plasma levels of insulin and glucagon before and after arginine infusion, and the immunohistochemical appearance of pancreatic A and B-cells.

Materials and Methods

Female Wistar rats with a body weight of about 200 g were fed Altromin rat chow and water *ad libitum*. They were housed in non-transparent cages with 5 animals in each, light was controlled to a 12 hr dark/light cycle. Temperature and humidity were kept constant. Six animals were injected with isotonic saline 5 ml/kg body weight twice weekly, and 30 animals were treated twice weekly with neutral red (2% aqueous solution 5 ml/kg body weight) half of the animals were treated with a solution of neutral red (Merck) recrystallised as described earlier (Okuda & Grollman 1966). Since the toxicity described by Okuda & Grollman (1966) did not occur from the brand of NR used the remaining animals were treated with untreated neutral red. All investigations were performed 24 hr after the last NR dose.

The animals were divided into groups of six for examination after treatment for 1, 2, 3, 4 and 5 weeks and six control animals were examined one day after examination of the 5 week group. After anesthesia with thiopental 100 mg/kg b.wt. intraperitoneally the animals were intubated and catheters (PE 50, Intramedic) were introduced into the left internal jugular vein for infusion and into the right common carotid artery for blood sampling. Prior to infusion of arginine 8 µg 100 g b.wt.⁻¹ min.⁻¹ (aqueous solution 100 mg ml⁻¹) blood glucose concentrations were measured by the glucose oxidase technique using a rapid glucose analyser (Yellow Springs, Ohio, U.S.A.) and blood taken into chilled tubes containing aprotinin and EDTA for determination of insulin and glucagon. Glucagon was extracted by ethanol according to Heding (1971). After infusion of arginine for 30 min. blood samples were taken as

described above for glucagon and insulin determination by RIA. For technical reasons five animals were excluded, 1 in week 1, 3 in week 2, 1 in week 4 and 1 in week 5, and blood glucose was measured only in one animal in week 2 and in no animals in week 3.

For histological investigations five other groups of rats were treated with neutral red and one with placebo as described above. The rats were anaesthetized with diethyl ether after 1, 2, 3, 4, and 5 weeks treatment with neutral red (10 animals in each group) and with placebo (5 animals) and their pancreas close to the spleen were removed for immediate fixing in Bouin's fluid for 4 hr followed by paraffin embedding. For morphological examination sections were stained with haematoxylin and eosin or with aldehyde fuchsin. For immunohistological investigation 10 micron sections were deparaffinized and treated first with a solution of 0.5% H₂O₂ in methanol for 30 min., then with 10% pig serum in PBS for 30 min., in order to minimize unspecific enzyme binding. The sections were then incubated for 20 min. with either guinea pig anti-insulin diluted 1:10000 or with rabbit anti-glucagon diluted 1:2000. To stain the insulin or the glucagon a 30 min. incubation with pig anti-guinea pig or pig anti-rabbit serum was performed. The reaction product was visualized using 3-amino-9-ethyl-carbazole (AEC) as a chromogen for 15 min. Ten mg of AEC was dissolved in 2.5 ml dimethylformamide and 47.5 ml 0.1 molar acetate buffer was added. H₂O₂ was added to a final concentration of 0.03%. Finally the slides were counterstained for 30 sec. with Mayer's haematoxylin in water, blued in tap water and mounted in Aquamount.

Sources of chemicals. Anti-insulin and anti-glucagon were supplied from Hagedorn Research Laboratory, Gentofte, Denmark. Pig serum and peroxidase labelled anti-glucagon were supplied from DAKO PATTS, Copenhagen. The batch of neutral red apparently did not possess the unwanted toxic properties occurring from some preparations (Okuda & Grollman 1966) since body weight increased identically in control animals and animals treated with either recrystallized dye or the stock dye. Therefore animals treated with the two preparations of NR are considered one group.

Statistical analysis. Comparison of groups was performed by a double-sided Student's t-test at a 0.05 level of significance.

Results

Twenty-four hr after the second administration of neutral red (NR) a two fold increase in plasma glucagon concentrations was reached and maintained during 5 weeks of treatment ($P < 0.05$, table 1). Plasma insulin concentrations showed a two fold increase compared to control rats (table 1). There were no changes in blood glucose concentrations, $P > 0.05$, control values being 4.3 ± 0.2 mmol/l.

During the 5 weeks of NR treatment glucagon/insulin

ratio increased, a statistical significant two fold increase ($P < 0.05$) was reached after 5 weeks of treatment (table 1).

Infusion of arginine for 30 min. increased plasma glucagon concentrations 10-fold ($P < 0.001$) in control rats. After NR treatment for two weeks arginine infusion only increased plasma glucagon concentrations 2-fold ($P < 0.05$), unstimulated glucagon levels still being higher ($P < 0.05$) than control values. After 5 weeks NR treatment a 10 fold increase in plasma glucagon concentrations after arginine infusion was regained ($p > 0.05$, table 1).

Arginine infusion increased plasma insulin concentrations 10-fold as expected (Gordin *et al.* 1977) in control rats. In the NR treated animals (1, 2, 3 and 4 weeks) arginine increased insulin concentrations considerably more ($P < 0.05$) than in control rats. In accordance with the hampered glucagon response and increased insulin response to arginine infusion after NR treatment glucagon/insulin ratio was decreased after one and two weeks NR treatment, arginine infusion did not change glucagon/insulin ratio in the control animals. After 5 weeks NR treatment the ratio after arginine stimulation was the same as in the controls (table 1).

Infusion of arginine increased blood glucose concentrations in control from 4.3 ± 0.2 (N=6) to 5.8 ± 0.4 mmol/l, slightly higher blood glucose concentrations were found in NR treated rats after arginine infusion.

On histological examination no difference could be demonstrated in the number or the size of the islets of Langerhans neither between pancreata from NR treated animals and untreated control animals, nor between the different groups of NR treated rats. None of the islet cells in the NR treated animals showed signs of atrophy or necrosis. However, while the contents of insulin in the B-cells was fairly constant the quantity of glucagon was conspicuously lowered in islets from animals treated with neutral red for one or two weeks. After treatment with NR for three or four weeks the amount of glucagon apparently was restored, and after five weeks the A-cells not only contained more glucagon than the untreated controls, but the relative number of A-cells within the islets had increased. Immunohistochemical reaction with glucagon in the islets of Langerhans from a control rat, and from rats treated with NR for 1 and 5 weeks is shown in figs. 1, 2 and 3.

Table 1.

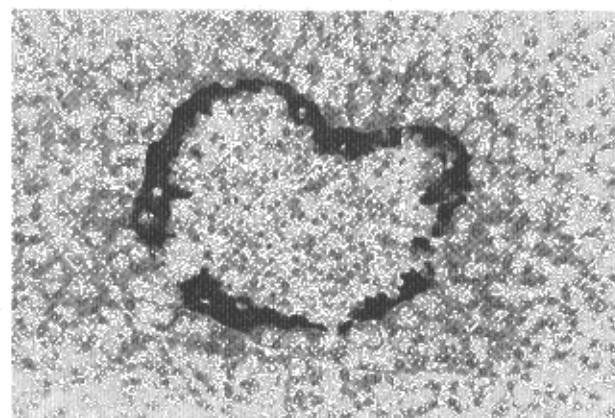
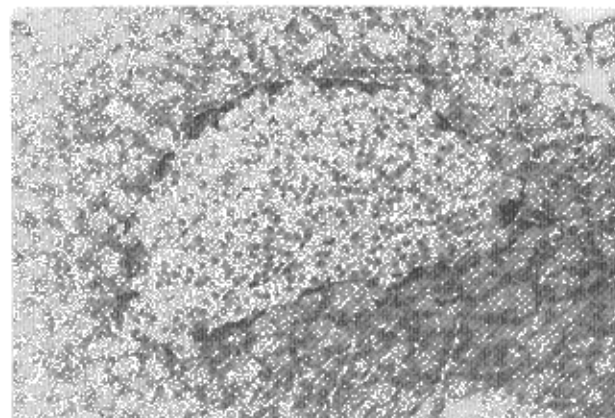
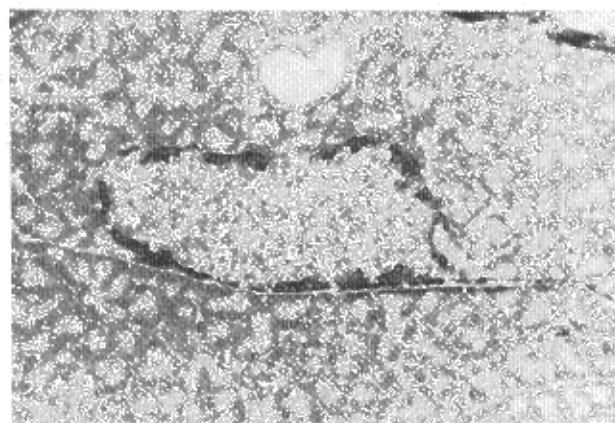
Plasma glucagon (ng/l), insulin (μ g/l), and glucagon/insulin ratio before and after 30 min. stimulation with arginine in rats pretreated with neutral red twice weekly.

	Control (n=6)	1 week (n=5)	2 weeks (n=3)	3 weeks (n=6)	4 weeks (n=5)	5 weeks (n=5)
Glucagon (G)	128 ± 24	309 ± 44*	192 ± 12*	168 ± 14	306 ± 39*	370 ± 44*
after arginine	1118 ± 395	470 ± 95	358 ± 104	803 ± 99	1265 ± 359	1501 ± 228
Insulin (I)	2.12 ± 0.20	4.20 ± 0.44*	2.88 ± 1.00	4.00 ± 0.44*	3.44 ± 0.84	3.00 ± 0.36*
after arginine	21.2 ± 2.4	56.0 ± 11.5	75.2 ± 11.8*	78.3 ± 16.4*	58.4 ± 16.5*	34.8 ± 13.2
G/I	64.8 ± 15.7	74.1 ± 7.9	84.8 ± 27.0	45.2 ± 7.2	110.9 ± 27.8	133.1 ± 25.5*
after arginine	56.6 ± 8.7	9.7 ± 2.6*	4.9 ± 1.5*	15.9 ± 6.2*	26.4 ± 6.4*	54.0 ± 10.5

Values indicate mean ± S.E.M., n is given in brackets, *denotes $P < 0.05$ t-test versus control group

Discussion

The present study shows that long term administration of neutral red (NR) to rats leads to hyperglucagonaemia and possibly a slight hyperinsulinaemia with normoglucaemia in accordance with the earlier suggestion that NR treatment leads to release of glucagon from the pancreatic α cells



Figs. 1, 2 & 3. Immunohistochemical localization of glucagon in the islets of Langerhans. The reaction product is located in the peripheral part of the islets in the control animal (fig. 1) as well as after treatment with neutral red for 1 week (fig. 2) or for 5 weeks (fig. 3). After neutral red treatment for 1 week the glucagon amount in the islet seems to be lowered while the result of treatment for five weeks is that the A-cells not only now are veritably loaded with glucagon, but their relative number has increased. Magnification $\times 450$.

(Okuda & Grollman 1966; Nevis *et al.* 1968). Furthermore it demonstrated that chronic NR administration inflicts a temporary deficiency in A-cell function characterized by a severely restricted ability to release glucagon upon stimulation, in this study arginine infusion.

After 1-2 weeks of NR treatment, the arginine induced plasma glucagon concentration increase was only half of that in the control rats, plasma insulin was higher and thereby the glucagon/insulin ratio decreased. After that interval there was no change in the glucagon/insulin ratio before arginine infusion, suggesting normal insulin sensitivity and reduced ability to secrete glucagon. After 5 weeks of NR treatment glucagon/insulin ratio was elevated which suggests decreased insulin sensitivity, at the same time arginine induced increase in glucagon and insulin plasma concentration was normalized resulting in a glucagon/insulin ratio identical to the control situation.

The effect of NR on insulin levels was small. After all periods of NR treatment mean insulin concentrations were higher after than in the control group. Although not statistically significant after all treatment intervals, insulin levels were moderately elevated from NR treatment, presumably as a response of the hyperglucagonemia (Unger 1985).

CoCl₂ has been demonstrated to have a similar toxicity as NR on the A-cells (Håkanson *et al.* 1974) after short term treatment as evaluated by electron microscopy and microspectrophotometry. Whether a similar compensatory effect after prolonged intoxication can be produced by this toxin has not been investigated.

The toxic effects of NR on pancreatic A-cells can be summarized as follows: Acute administration leads to a release of glucagon from A-cell granula and subsequent hyperglucagonaemia. Continued administration is followed by a sustained release of glucagon and sustained hyperglucagonaemia. Additionally the ability to further release of glucagon by stimulation e.g. by arginine is temporarily restricted. Prolonged administration (5 weeks) of NR leads to hyperglucagonaemia and the ability to respond to arginine with further glucagon release is regained. This condition is associated with morphological signs of hypertrophy and hyperplasia of the A-cells. The changes in plasma glucagon concentrations are accompanied by increased plasma insulin concentrations which probably are compensatory. The NR treated rat thus is a useful model for the study of metabolic processes with hyperinsulinemia/hyperglucagonaemia with or without restricted ability to further glucagon release. However, it should be emphasized that NR does not do to the A-cells what alloxan and streptozotocin does to the B-cells.

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