The pharmacological relevance of vital staining with neutral red

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Summary. The uptake of neutral red into the renin-containing juxtaglomerular granules does not inhibit the release of renin either in basal or in stimulated states of renin secretion. The vasodilating effect of neutral red may be due to a non-specific binding to noradrenaline-receptors in the vascular smooth muscle cells.

Evidence has been presented that the granules in the juxtaglomerular (JG) cells are the site of renin synthesis and storage[3]. Neutral red (NR) is taken up by, and concentrated in, the JG granules[3,4]. Striking ultrastructural changes have been observed in the JG granules of rats 1 h after the i.v. administration of NR[5]. However, no data are available about whether the uptake of NR may also induce functional changes in the juxtaglomerular cells. The present investigation was undertaken to correlate the structural and possible functional effects induced by NR.

Material and methods. Male Sprague-Dawley rats, weighing 170–200 g, received various doses of NR (CI 50040, Chroma), dissolved in 0.9% NaCl and administered either i.v. or i.p. The uptake of NR into the JG granules was controlled by fluorescence microscopy. Sections were prepared according to the Falck-Hillarp technique[6] to demonstrate NR and the formaldehyde-induced fluorescence of catecholamines simultaneously.

The activity of the renin-angiotensin system was assessed by measuring the plasma concentration of angiotensin II (A II) by direct radioimmunoassay[7]. Renin release was stimulated either by bleeding (1% of b.wt, 30 min before the rats were killed) or by the i.v. injection of furosemide in a dose of 50 mg/kg b.wt. Systolic blood pressure was measured by tail plethysmography under light ether anaesthesia. Packed cell volume (PCV) was determined in heparinized microtubes, centrifuged for 10 min at $10{,}000 \times g$. The effect of i.v. administered NR on the blood pressure was recorded under light thiobarbital anaesthesia by a catheter placed in one femoral artery and connected to a strain gauge. The direct vascular action of NR was studied in the isolated hindlimb preparation of the rat[8] perfused with a medium that contained either $10^{-5.5}$ M/l L-noradrenaline or 90 mM KCl as vasoconstrictor.

Results. 2 h after the i.p. administration of 5×10^{-4} M NR/kg b.wt, the dye had accumulated in the JG granules, which showed an intense red vital staining in mixed light. In UV-light, a strong brick-red fluorescence was observed in the JG granules, but not in other parts of the JG apparatus. The characteristic blue-green formaldehyde-induced fluorescence of noradrenaline was clearly visible in the adrenergic plexus of the juxtaglomerular arterioles.

In spite of the accumulation of NR in the renin-containing JG granules, the plasma concentration of A II remained unchanged (104.75 ± 10.54 pg A II/ml vs 95.12 ± 13.65 pg A II/ml of control rats). No changes in haemoconcentration were observed. The administration of NR also had no effect on the stimulated release of renin (figure 1). After bleeding, the increase in the plasma concentration of A II was similar in control rats and in those that had received NR prior to bleeding (173 ± 15.95 pg A II/ml vs 178 ± 38.09 pg A II/ml). The increase in the plasma concentration of A II induced by furosemide was even enhanced by NR (389.0 ± 39.42 pg A II/ml vs 206.75 ± 29.43 pg A II/ml). After bleeding, PCV fell from 42 to 37%, whereas, after furosemide, it rose from 42 and 40% to 43 and 44%, respectively.

Neutral red has a potent vasodilator effect. After 5×10^{-6} M NR i.v., the blood pressure fell transiently by about 30 mm Hg. In the isolated hindlimb preparation, NR already began to inhibit the vasoconstriction caused by L-noradrenaline at a concentration of 10^{-7} M NR/l perfusion fluid, and the reduction of vascular resistance was proportional to the dosage of NR. However, when identical vascular resistance was induced by 90 mM KCl in the perfusion fluid, NR began to reduce the vascular resistance only at 10^{-5} M NR/l perfusion fluid, and the maximal reduction of vascular resistance at the highest NR concentration was not comparable to that observed in the case of L-noradrenaline-induced vasoconstriction (figure 2). The i.v. administration of NR in doses up to 2.5×10^{-4} M/kg b.wt did not affect the formaldehyde-induced catecholamine fluorescence in the juxtaglomerular arterioles and produced no light microscopic changes in the macula densa. Higher doses of NR (5×10^{-4} to 10^{-3} M NR/kg b.wt i.v.), however, abolished the catecholamine fluorescence in the adrenergic plexus of the juxtaglomerular arterioles and caused a selective nuclear staining in macula densa cells.

Discussion. Neutral red is a relatively non-toxic, weakly cationic dye, which was introduced as a vital stain by Ehrlich in 1894[9]. The ubiquitous distribution of NR in the organism is explained by non-ionic diffusion – a mechanism by which weak acids and bases pass through lipid membranes in a non-ionized form. The molecular binding mechanism between NR and cellular components is not known. NR accumulates in lysosomes. The uptake of NR by the JG granules[4] has been correlated with their lysosomal nature. JG granules contain renin (a proteolytic enzyme)[10] and acid phosphatase[11-13]. The cytochemical composition of the granular matrix is also similar to that of lysosomes[14].

The ultrastructural changes observed in the JG granules after the uptake of NR[5] might suggest an alteration of their enzymatic function. The results of our study, however, point out that the binding of NR to JG granules does not cause a functional disturbance of the renin-angiotensin system. Renin release, unaffected by the uptake of NR into the

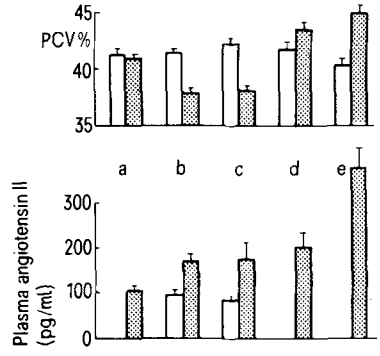

Fig. 1. Effect of neutral red on PCV and plasma A II concentration. Packed cell volume and plasma angiotensin II concentration before □ and after ■ treatment with a) neutral red (5×10^{-4} M/kg i.p.), b) bleeding (1% of the body weight, 30 min before sacrifice), c) neutral red + bleeding, d) furosemide (50 mg/kg i.v.), e) neutral red + furosemide.

granules, is in keeping with the observation that the enzymatic activity of lysosomes is not changed after vital staining with NR[15]. NR has a potent vasodilator action in relatively low doses. The results of our hindlimb-perfusion experiments show that the inhibition of noradrenaline-induced vasoconstriction by NR is the probable mechanism for the vasodilation. NR has a special capacity to accumulate in the granules of the APUD endocrine cells[16], which also bind catecholamines and their precursors[17,18]. It is conceivable that NR has an affinity to other catecholamine-binding sites, too. Thus, NR can displace noradrenaline from its binding sites in vascular smooth muscle cells.

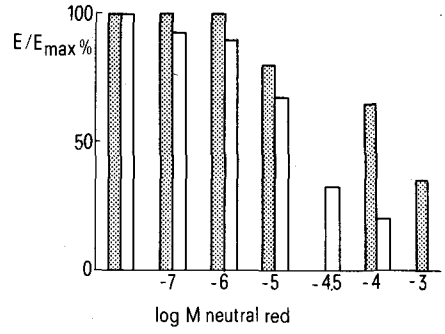

Fig. 2. Effect of neutral red on the perfusion pressure of isolated hindlimbs. White columns represent the effects of neutral red concentrations on the perfusion pressure produced by $10^{-5.5}$ M/l L-noradrenaline. Dotted columns represent the effects of neutral red concentrations on the perfusion pressure produced by 90 mM/l KCl.

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2 This work was supported by the German Research Foundation within the SFB 90 'Cardiovasculäres System'.
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Skeletal muscle capillary densities during reactive hyperemia

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Summary. Reactive hyperemia was induced in hindlimbs of rats by occlusion of the femoral artery. Using fluorescein dye as a peripheral vascular marker, we observed that there was an increase in the number of flowing capillaries supplying the muscle fibres following release of the occlusion. The results indicate that the number of flowing capillaries is not dependent on the duration of occlusion (2-10 min).

Reactive hyperemia is defined as the increase in blood flow which occurs immediately following the release of complete arterial occlusion. Krogh[1], using a method of intravitam injection of India ink, observed that reactive hyperemia in skeletal muscle was associated with an increase in the number of flowing capillaries. More recently, Burton and Johnson[2], using a dual slit photometric system to measure red blood cell velocity profiles, suggested that the increase in blood flow following occlusion release is due to an augmentation of blood flow through previously flowing capillaries rather than an increase in the number of flowing capillaries. In the experiments reported here, we measured capillary densities following arterial occlusion in the musculature of rats using fluorescein dye as a peripheral vascular marker. In addition, we investioated the relationship between occlusion duration and capillary density since both the magnitude of flow response and duration of hyperemia have been shown to be related to the duration of cessation of flow[3,4].

Materials and methods. Adult male rats (500-750 g) were anesthetized with sodium pentobarbital (i.p., 35 mg/kg) and one of the external jugular veins was cannulated with polyethylene tubing. The gracilis muscle of each hindlimb was exposed. The femoral artery of the left hindlimb was dissected free and occluded for either 2, 6, 8 or 10 min. The right hindlimb served as the control since no elevation in blood flow through the contralateral vessel during reactive hyperemia has been reported[5]. At the end of the occlusion period, blood flow was returned to the left hindlimb. 15 sec after release of the arterial occlusion, a 6.0% solution of sodium fluorescein (300 mOsmols; 0.005 ml/g b.wt) was injected via the jugular cannula. 8 sec after the completion of injection, the gracilis muscle of each hindlimb was frozen in situ by the use of copper plated forceps precooled in liquid nitrogen (−196 °C). These timed intervals before freezing were determined to be optimal for all durations of occlusion suggesting that the time to peak capillary response was similar for all occlusion intervals under these conditions. Following freezing, each muscle was quickly excised from the animal and placed in separate dewars of liquid nitrogen. The frozen muscles were removed from the liquid nitrogen and placed in a cryostat (−30 °C; International Model CTV Microtome Cryostat). A tissue block (6×2×1 mm) was cut from the center of the muscles and mounted for sectioning so that the axial ends of the muscle were cut. After sectioning, the tissue sections (6-12 µm) were freeze-dried at pressures between 10^{-4} and 10^{-5} Torr for 14-16 h (Thermovac cryopump, TEFD-G2). Following