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COMPARATIVE EVALUATION OF THE *in vitro* EFFECTS OF HYDRALAZINE AND HYDRALAZINE ACETONIDE ON ARTERIAL SMOOTH MUSCLE

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1 Dose-response relationships to K^+ were determined in isolated strips of rabit aorta.

2 K^+ contractures were induced by 30 mM K^+ in paired strips from individual animals. The effects of hydralazine and hydralazine acetone hydrazone (hydralazine acetonide) on these contractures were studied.

3 Hydralazine and hydralazine acetonide both produced dose-dependent decreases of K^+-induced tone. Threshold concentrations for hydralazine were $11.89 \pm 4.5 \times 10^{-5}$ M (mean ± s.d.) and for hydralazine actonide $9.7 \pm 4.6 \times 10^{-5}$ M ($0.5 < P < 0.4$).

4 The magnitude of the effect of hydralazine acetonide was greater than that of hydralazine at all concentrations above threshold, as reflected in a significant difference ($P < 0.05$) in the slopes of dose-response curves to the two treatments. The vasodilator effects of hydralazine and the acetonide were terminated by washout of the bath.

5 The differences in effect were not due to instability of hydralazine under *in vitro* conditions.

6 It is concluded that hydralazine acetonide has intrinsic activity on vascular smooth muscle which differs significantly from that of the parent compound and that this may contribute to the hypotensive effects which follow administration of the parent compound.

Introduction

The vasodilator, hydralazine, is a widely used antihypertensive agent. However, its clinical application has been limited by dose-related, immunologically mediated, side effects (Koch-Weser, 1976). Metabolism of hydralazine seems important in the incidence of these side effects, since they occur with much greater relative frequency in patients in the slow acetylator phenotype category (Perry, 1973). Thus, although it was shown in earlier clinical use that the drug produced increasing effects up to doses as high as 1600 mg/day (Perry, 1973), at the present time dose limits of 200–300 mg/day are recommended (Koch-Weser, 1976).

Pharmacodynamic studies in humans have shown that the duration of the hypotensive effects of hydralazine is greater than expected relative to its concentration in blood (O'Malley, Segal, Israili, Boles, McNay & Dayton, 1975). It has been suggested that this discrepancy may be due to the existence of a 'deep compartment' in which persistence of hydralazine or hydralazine metabolites exceeds the persistence of hydralazine itself in plasma. Pharmacokinetic studies by O'Malley and co-workers using [^{14}C]-labelled hydralazine are consistent with either of the above possibilities (O'Malley *et al.*, 1975).

The metabolic fate of hydralazine and the biological activity of its products is of scientific interest and practical importance due to the possibility that active metabolites may mediate the prolonged pharmacodynamic effects of the parent substance. Further interest stems from the possibility that the metabolites causing hypotensive effects may differ from those mediating immune toxicity.

The development of techniques utilizing combined gas-liquid chromatography and mass spectrometry (g.l.c.-m.s.) (Haegele, Skrdlant, Robie, Lalka & McNay, 1976) has allowed quantitiative study of metabolism of hydralazine in the rat. Studies on

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anaesthetized animals, in which urine was collected over a period of 4 h, showed that the major pathways of *in vivo* metabolism in this species are represented by acetylation, hydrolysis of the hydrazine group, ring hydroxylation and the formation of hydrazones between ketone bodies and hydralazine (Haegele *et al.*, 1976). Further studies on conscious rats have indicated that the acetone hydrazone of hydralazine (acetonide) is a persistent metabolite of hydralazine after the parent compound is no longer detectable in body fluids (Haegele, McLean, Skrdlant, du Souich & McNay, unpublished). It was thus of interest to assess the pharmacological effects of this metabolite, and to contrast these with those of hydralazine.

Preliminary studies indicated that hydralazine acetonide produces hypotensive effects in conscious unrestrained rats (McLean, Haegele, du Souich & McNay, 1977). Because of difficulties in making accurate comparisons of vasodilator activity under *in vivo* conditions and because of the possibility that the activity of hydralazine acetonide stemmed from metabolic reconversion to hydralazine, it was considered important to assess *in vitro* effects.

Methods

Aortic strip preparation

Aortic strips were prepared according to the method of Carrier, Wedell & Barron (1977).

New Zealand Large White rabbits (body weights 1.5–2 kg) were stunned and exsanguinated. The thorax was opened and the aorta was removed from the arch to the diaphram. This was dissected clean of adipose tissue and connective tissue down to the adventitia in oxygenated Locke solution in a dissection dish. The aorta was then cut longitudinally opposite the line of origin of the intercostal branches; it was pinned out flat and transverse strips were cut with razor blades.

Strips were mounted on preparation holders, placed in isolated organ baths filled with Locke solution (composition mM: Na^+ 154, K^+ 5.6, Ca^{2+} 2.17, Mg^{2+} 0.024, HCO_3^- 6.0 and glucose 2.77) and attached to Grass FTO_3 tension transducers used with an Offner polygraph. Strips were equilibrated under 1 g tension for 1 h before study. Responses to K^+ were quantitated over the range 5.6 to 40 mM K^+ by cumulative addition of KCl to the bath.

Hydralazine and hydralazine acetone hydrazone (acetonide)

Hydralazine (1-hydrazino-phthalazine hydrochloride) was obtained commercially (Sigma Corp.) and hydralazine acetonide was synthesized as described previously (Haegele *et al.*, 1976). 1-Hydrazino-phthalazine-hydrocyloride (395 mg; 2 mmol) was dissolved in 2.5 ml of acetone and allowed to react for 1 hour. The solvent was evaporated and the slightly yellow material was dried *in vacuo*. The yield was 411 mg or 99% theoretical yield. The products of synthesis were assessed by g.l.c.-m.s. as described below.

Combined gas-chromatographic/mass spectrometric techniques

A Hewlett-Packard gas chromatograph-mass spectrometer-computer system (Model No. 5710A, 5980A, and 5933A) was used. The column was a 3 ft × 4 mm i.d. glass coil packed with 3% OV-17 on Chromosorb W, 80–100 mesh with helium carrier gas at a flow-rate of 40 ml/minute. A column temperature program of 4°/min from 170 to 250°C was used. The injector port temperature was 250°C and the interface of gas chromatograph to mass spectrometer consisted of a Hewlett-Packard membrane separator. Mass spectra were obtained by the electron impact mode of ionization, with an ionization energy of 25 eV; ion source temperature was 200°C. The mass spectrometer was interfaced to a Hewlett-Packard Model 5933A computer system which controlled the scan of the instrument and processed and displayed mass spectra.

Testing for vasodilator effects

Because at least 3 comparable strips were available from any one animal it was possible to allocate one strip to control and one to each of the test substance treatments. Hydralazine and hydralazine acetonide were cumulatively added to the individual baths over the dose range 10^{-6} to 5×10^{-4} M, and the relaxation induced in the strips was measured. Doses of hydralazine in excess of 5×10^{-4} M were not studied because of the difficulty in solubilizing appropriate stock solutions.

Assay of bath fluid

Bath fluid was taken after metabolite testing and extracted into organic solvents as detailed previously (Haegele *et al.*, 1976). Samples were then assayed using the g.l.c.-m.s. techniques described above.

Results

Responses of aortic strips to K^+

Dose-response relationships to K^+ were studied in 6 strips from 5 animals. Threshold concentrations were 8.0 ± 1.9 mM (mean ± s.d.; $n=6$), ED_{50}

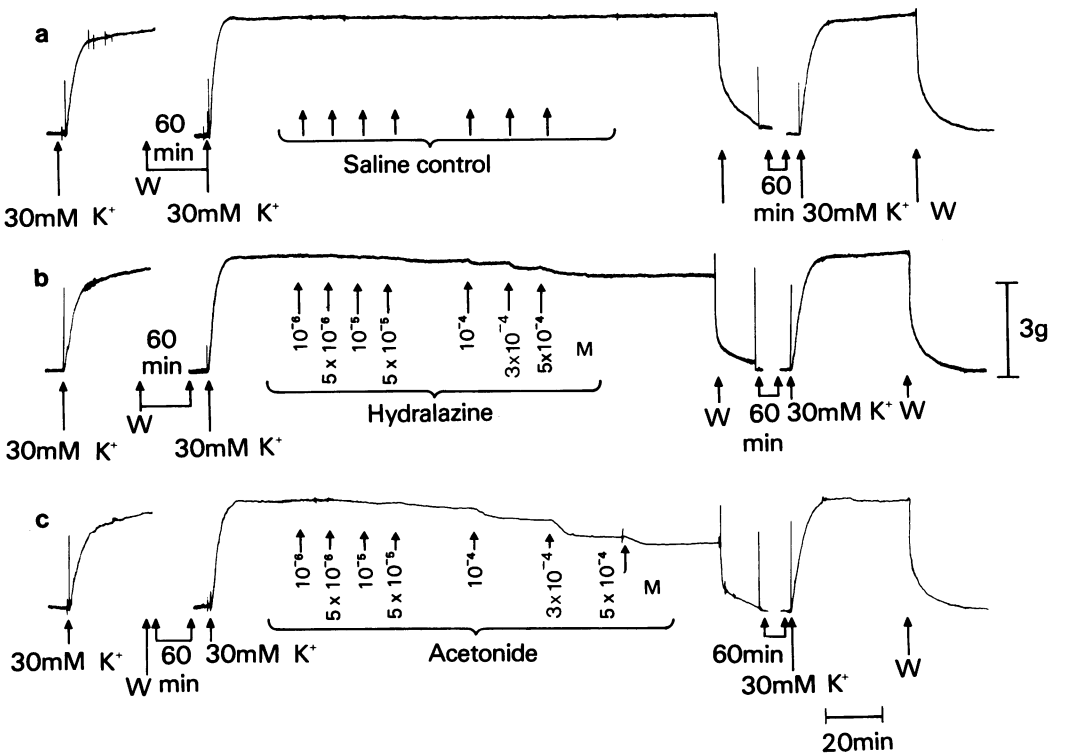

Figure 1 Illustration of K^+-induced contractures in paired aortic strips from one rabbit, and the effects of test vasodilators on induced tone. After equilibration, test contractures were induced to establish the equivalence of the preparations. The effects of metabolites were tested on the second contracture, one paired strip maintained as control (a). Test vasodilators were added cumulatively to the bath in parallel over the test range (b and c). The baths were washed out as indicated (W), then a final contracture was induced.

20.3 ± 1.7 mM. Maximal contractions were elicited by 38.8 ± 2.0 mM.

Sustained submaximal contractures were induced by replacement of normal bath fluid by 30 mM K^+ Locke solution prepared by Na^+/K^+ substitution (see Figure 1a). The absolute tension generated by strips varied considerably between animals (1.85–3.65 g), however the responses of strips from any one animal showed little variation, e.g. <2.9% in all 6 sets of strips in which drug application was studied, as illustrated in Figure 1.

The 'within animals' variation was assessed in a further 11 aortae (44 strips). One way analysis of variance (Steel & Torrie, 1960) showed that the differences observed could be accounted for by random variation ($f=0.034$, $P=0.9908$).

Validation of products of acetonide synthesis

Testing of the produce of acetonide synthesis yielded the mass spectrum of the acetone hydrazone with the molecular ion at m/e 200. Loss of a methyl radical then gives rise to the base peak at m/e 185. No impurities were found to be present in the sample.

Effects of hydralazine and hydralazine acetonide on K^+ contractures

Both acetonide and hydralazine caused dose-dependent relaxation of K^+ contractures (Figure 1). Doses of acetonide in the range 1 to 2×10^{-3} M caused a complete abolition of induced tone; comparisons were limited to the range 10^{-6} to 5×10^{-4} M (see Methods).

The results obtained in 6 sets of aortic strips are shown in Figure 2. Threshold values were determined for each treated strip by least squares linear extrapolation of the line of best fit. These were $11.89 \pm 4.5 \times 10^{-5}$ M (mean ± s.d.) for hydralazine and $9.7 \pm 4.6 \times 10^{-5}$ M for hydralazine acetonide. These differences were not significant ($0.5 > P > 0.4$). The acetonide produced greater degrees of relaxation than hydralazine itself and these differences became increasingly significant as the bath concentrations

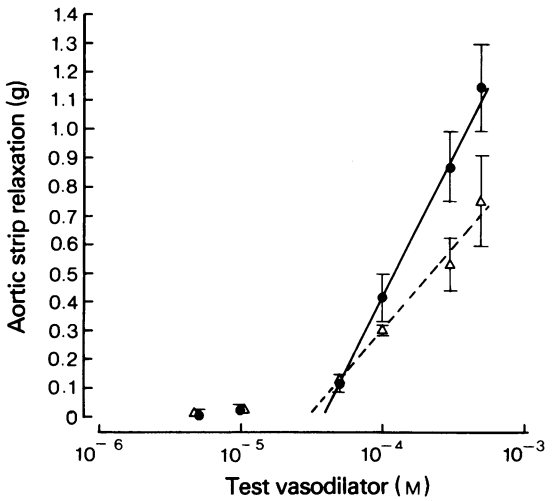

Figure 2 Cumulative dose-relaxation relationships to hydralazine and hydralazine acetonide on K^+-induced contractures of paired rabbit aortic strips. Each point represents the mean of determinations on 6 strips, the vertical lines represent the standard deviations. (△). Hydralazine; (●) hydralazine acetone hydrazone (acetonide).

were increased ($0.2 < P < 0.0.3$, 10^{-4} M; $P < 0.05$, 3×10^{-4} M; $P < 0.02$, 5×10^{-4} M). The slopes of the dose-response relationships to the acetonide were significantly greater than those to hydralazine ($P < 0.05$) reflecting the similarity in threshold doses and the increasing differences in response with increasing dose.

The effects of both compounds were reversible on washout, as baseline tonus was not altered, and further contractures to K^+ in the treated strips did not differ significantly from further contractures in paired control strips as illustrated in Figure 1.

Analysis of bath fluid

One possible explanation of the differences between the responses to higher doses of hydralazine and acetonide was the known instability of hydralazine under neutral and alakaline conditions (Schulert, 1961). To test for this possibility, samples of bath fluid were removed after full relaxant effects had been produced by 5×10^{-5} M and 5×10^{-4} M hydralazine. These were extracted and assayed for the presence of phthalazine and/or phthalazinone by g.l.c.-m.s. techniques described previously (see Methods). There was no evidence of phthalazine or phthalazinone production under the conditions of these experiments, indicating that differences in effects were based on real differences in pharmacological activity.

Discussion

The development of a transverse strip preparation of aortic smooth muscle allows the simultaneous comparison of several vasoactive agents in paired strips, in contrast to the conventional spiral strip preparation. This has been exploited in this study to allow quantitative comparisons between the vasodilator potency of a metabolite of hydralazine and the parent compound. The results establish that there are no significant differences in threshold, but that there are significant differences in effect above threshold. This is compatible with the conclusion that hydralazine acetonide and hydralazine activate a common receptor for which they have similar affinity, but that they show differing intrinsic activities. However, in the absence of comparisons up to 100% of maximum vasodilator effect (presumably complete loss of K^+-induced tone), any conclusion related to intrinsic activity remains speculative.

The effect of the vasodilators appeared to be reversed on washout, as K^+-induced contractures of the control strip did not differ from those of the treated strips at the end of the experiment. These results tend to suggest that there is a freely reversible association of drugs with vascular tissue which determines vasodilator activity. This possibility is of significance because of previous evidence that hydralazine and/or metabolites preferentially concentrate by 'tight' binding within blood vessel walls (Perry, Comens & Yunice, 1962; Moore-Jones & Perry, 1966; Wagner, 1973; Keberle, Faigle, Hedwall, Riess & Wagner, 1973), in an active form (Moore-Jones & Carmody, 1966). It has been thought likely that this tissue binding provides the basis for pharmacokinetic 'deep compartments' (Lesser, Israili, Davis & Dayton, 1974; O'Malley *et al.* 1975). These 'sequestered' compounds were considered to provide one possible mechanism of mediation of the prolonged effects of hydralazine (Keberle *et al.*, 1973; O'Malley *et al.*, 1975). The results described here would tend to discount the functional significance of 'irreversibly' bound ^{14}C-labelled compounds found in blood vessel walls following the administration of [^{14}C]-hydralazine to intact animals.

This work constitutes the first report that a metabolite of hydralazine has vasodilator effects under *in vitro* conditions, and establishes that this compound is more active than the parent compound. Given that acetonide is a persistent product of metabolism, these results relating to its potency as a vasodilator tend to suggest that the acetonide could be an important mediator of the unexplained, prolonged hypotensive effects of hydralazine. If it could be shown that the acetonide, or other active metabolites, differ in their propensity for producing immunologically mediated side effects, the therapeutic index of hydralazine-related drugs could be materially improved.

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