An investigation of sex-linked differences to the toxic and to the pharmacological actions of difenacoum: studies in mice and rats

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We have investigated the actions of the coumarin anticoagulant, difenacoum, in male and female rats and mice. In our first experimnt difenacoum (0.5 mg kg^{-1}) killed 50% of male mice within 9 days of its administration, whereas no female mice died during this study. In a second group of experiments, the anticoagulant effect of difenacoum in male and female rats was determined. Under resting conditions, the prothrombin complex activities (PCA) of male and female rats were not significantly different. Over the first 24 h after administration of difenacoum (0.4 mg kg^{-1} i.p.), there was a monoexponential fall in PCA in both sexes. However, 6, 12 and 24 h after difenacoum, the PCA in male rats was significantly ($P < 0.05$) lower than in female rats. PCA began to recover over the subsequent 48 h in both sexes, during which time there was marked variability in recovery in female rats. The difference between the onset of action of difenacoum in male and female rats did not appear to be due to a greater rate of elimination of the drug in female rats, since the plasma concentrations of difenacoum 24 h after its administration were the same in both sexes. The concentration of vitamin K_1 in rat liver was also investigated. Vitamin K_1 levels were 35.1 ± 18.6 ng (g liver)$^{-1}$ (male), and 29.4 ± 5.4 ng (g liver)$^{-1}$ (females) in control rats, but 24 h after difenacoum, vitamin K_1 levels were either very low, or undetectable in all rats. It is, therefore, possible that the difference between the responses to difenacoum in males and females was due to a greater rate of breakdown of vitamin K-dependent clotting factor precursors in male rats and mice, compared with female rats and mice.

The influence of vitamin K_1 on the synthesis of clotting factors II, VII, IX and X can be studied by observing the effects produced after procedures that deplete the biological availability of vitamin K_1. Two such interventions are the production of dietary vitamin K deficiency, and the use of coumarin anticoagulants. It has been shown that the fall in the activity of clotting factors induced by dietary vitamin K deficiency is more rapid in male rats than in female rats (Johnson et al 1960). Indeed, female rats appear to be protected against the anticoagulant effects of a dietary deficiency in vitamin K by the presence of oestrogenic hormones (Mellette & Leone 1960). However, the mechanisms by which oestrogens protect against vitamin K deficiency remain uncertain (Rama Rao et al 1964; Matschiner & Bell 1973; Siegfried et al 1979).

Coumarin anticoagulant drugs inhibit the enzymes vitamin K_1 2,3-epoxide reductase, and vitamin K_1-reductase (Matschiner & Willingham 1974; Whitlon et

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al 1978). This disrupts the vitamin K_1-epoxide cycle, thereby reducing, or abolishing, clotting factor synthesis. However, as yet, there is little available evidence concerning the possible difference between the pharmacodynamic actions of coumarin anticoagulant drugs in males and females. In the present study, we have investigated the actions of the coumarin anticoagulant, difenacoum, in male and female mice and rats. Although warfarin is often used in experimental studies, difenacoum was chosen for the present work because it has both greater potency, and a longer duration of action than warfarin (Park & Leck 1982). This allows short-term pharmacodynamic changes resulting from differences between males and females to be studied in the absence of marked pharmacokinetic effects.

Methods

Male and female Random BKW mice were obtained from Bantin and Kingman Ltd, Hull. A total of 28 male, and 24 female, adult, age-matched Wistar rats were also used (weights 288 ± 6 g male; 246 ± 10 g female); rats were bred in the departmental animal unit. Difenacoum (3-(3-[1,1′-biphenyl]-4-yl-1,2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-2H-1-benzopyran-2-one; Sorex Laboratories, Widnes) was given intraperitoneally in polyethylene glycol 200; the volume of the vehicle was no greater than 0.1 mL. All results are expressed as mean $\pm$ s.e. mean, and statistical significance was determined using the Mann-Whitney U-test for non-parametrically distributed data. Four experiments were performed:

(i) *Toxicity of difenacoum in male and female mice.* More female mice were available than were male mice. Therefore, for this preliminary experiment a total of 12 male mice, and 20 female mice were used. A single dose of difenacoum (0.5 mg kg^{-1} i.p.) was given to each mouse, and the number of deaths that occurred per day were recorded over the subsequent 20 days.

(ii) *Measurement of prothrombin complex activity in male and female rats.* Twelve male, and 12 female Wistar rats were used. Blood samples were collected from the caudal artery into 3·8% trisodium citrate (10% citrate; 90% blood, by volume), whilst the rats were lightly anaesthetized with ether. Blood samples (0.6 mL) were taken at 0, 6, 12, 24 and 72 h after difenacoum (0.4 mg kg^{-1} i.p.). PCA was determined by the method of Quick (1957), and clotting time was

measured using a Schnitger and Gross coagulometer. Clotting times were related to % PCA using a standard curve derived from pooled, normal, citrated plasma that was diluted with citrated plasma taken from severely anticoagulated rats (clotting times $\geqslant 500$ s, indicating the absence of vitamin K_1-dependent clotting factors).

(iii) *Measurement of the plasma concentrations of difenacoum in male and female rats.* Seven male, and 7 female rats were used. Blood samples for the determination of plasma difenacoum concentrations were taken immediately before, and at 24 h after the administration of difenacoum. At $t = 0$ h, blood (2 mL) was taken from the caudal artery under light ether anaesthesia. At $t = 24$ h, cardiac punctures were performed under ether anaesthesia, and 8–10 mL blood were taken from each rat. The plasma concentrations of difenacoum were determined by HPLC (Breckenridge et al 1985).

(iv) *Measurement of the concentration of vitamin K_1 in rat liver homogenate.* Nine untreated male rats, and 5 untreated female rats were used in this study. Livers from the 7 male and 7 female difenacoum-treated rats (described above) were also used. To ensure that the treatments were similar in both groups, cardiac punctures were performed on control animals under ether anaesthesia, and 10 mL blood was removed from each rat. All of the animals were then killed by cervical section, and the livers removed and placed on ice before being stored at $-20\,°C$ until use.

Livers were homogenized in an equal volume of buffer (consisting of 405 mL 0.2 M Na_2HPO_4, 95 mL 0.2 M NaH_2PO_4, 500 mL distilled water, and 11.5 g KCl), using 5 strokes of a Potter-Elvejehem homogenizer equipped with a close fitting Teflon tip. A total of 3.5 mL of each of the homogenates was extracted by gentle tumbling for 20 min in acetone and hexane (2 and 5 mL, respectively), after the addition of an internal standard (menaquinone-6; MK-6). After centrifugation, the organic layer was removed and was concentrated in a vortex evaporator. The residue was then redissolved in 1.5 mL hexane and purified through preparative silica columns (Sep-Pak, Waters Associates), followed by normal-phase HPLC for timed collections of the vitamin K_1 and MK-6 fractions. Final concentrations of vitamin K_1 were determined using electrochemical detection (Coulochem electrochemical detector, model 5100A) equipped with a dual electrode cell (model 5011), containing two porous graphite electrodes in series (Environmental Science Associates). This assay was developed by Hart et al (1985).

Results

(i) *Toxicity of difenacoum in male and female mice.* Between day 4 and day 9 after the administration of a single dose of difenacoum (0.5 mg kg^{-1} i.p.), 6 of the 12 male mice died, but there were no further deaths between day 9 and the end of the experiment (20 days).

In the group of female mice (n = 20), no deaths were recorded over the duration of the experiment.

(ii) *Measurement of prothrombin complex activity in male and female rats.* Normal PCA was not different in female rats, compared with male rats. Within 6 h of the administration of difenacoum, PCA had fallen in both groups, and continued to fall over the first 24 h after drug administration, until nadirs of $2.8 \pm 0.6\%$ (male), and $4.8 \pm 1.3\%$ (female), of the normal PCA were reached; the nadirs of the fall in PCA in both male and female rats were significantly ($P \leqslant 0.05$) below control levels. At 72 h, PCA had recovered in both male and female rats, although it remained significantly ($P \leqslant 0.05\%$) below the level seen in the absence of difenacoum in both male and female rats (Fig. 1).

Six hours after the administration of difenacoum, PCA was significantly lower ($P \leqslant 0.05$) in male rats than in female rats ($38.9 \pm 2.5\%$ male; $53.6 \pm 6.3\%$ female). Likewise, after both 12 h (PCA $14.9 \pm 1.3\%$ male; $20.5 \pm 1.1\%$ female), and 24 h (see above), PCA was significantly ($P \leqslant 0.05$) lower in male rats than in female rats. The fall in PCA was monoexponential in both groups, but the half-life of decline of PCA was shorter in male rats (4 h 30 min) than in female rats (5 h 10 min).

The recovery of PCA appeared to be more marked in female rats than that seen in male rats ($10.0 \pm 2.7\%$ males; $42.3 \pm 18.6\%$ females). However, there was considerable inter-animal variability in the PCA recorded in the female rats at this point. Therefore, the difference in PCA was not significant.
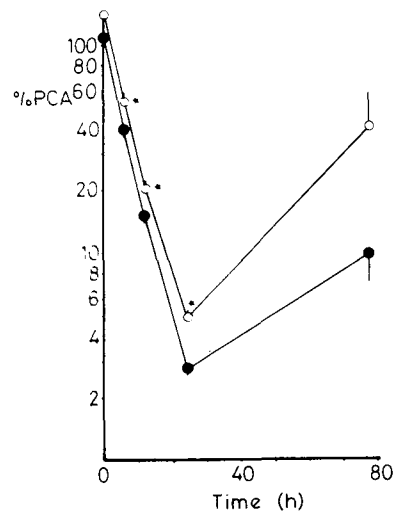

FIG. 1. Per cent prothrombin complex activity (PCA) over the first 72 h after the administration of difenacoum (0.4 mg kg^{-1} i.p.) in male rats (●), compared with female rats (○). Asterisks denote statistical significance between the sexes (Mann-Whitney U-test; $P < 0.05$).

(iii) *Measurement of the plasma concentrations of difenacoum in male and female rats.* Difenacoum was not detectable in control plasma. Twenty-four hours after administration of the drug, the plasma concentrations of difenacoum were the same in male rats (86·9 ± 21·8 ng mL^{-1}), compared with female rats (91·1 ± 18·7 ng mL^{-1}).

(iv) *Measurement of the concentration of vitamin K_1 in rat liver homogenate.* In control animals, vitamin K_1 was measurable in both male and female rats. However, whilst the concentrations of vitamin K_1 were similar in each of the female rats (mean 29·4 ± 5·4 ng (g liver)$^{-1}$), there was considerable inter-individual variation in the concentrations of vitamin K_1 seen in male rats (mean 35·1 ± 18·6 ng (g liver)$^{-1}$). This variability was consistent, since it was seen in an initial group of n = 5 male rats, and also in an additional group of n = 4 male rats. Since there was less variability in the female rats (n = 5), no further experiments were performed in female rats.

In the presence of difenacoum, vitamin K_1 was undetectable in female rats. In all but one of these rats, there was a large peak that eluted immediately before vitamin K_1. Low concentrations of vitamin K_1 (3·38 ± 2·2 ng (g liver)$^{-1}$) were detected in 3 out of 7 male rat liver homogenates, and there was no detectable vitamin K_1 in the remaining rats (limit of sensitivity 0·5 ng). In two of these latter rats, there was a large peak that eluted immediately before vitamin K; this peak had the same retention time as that recorded in 6 out of the 7 female rats.

Discussion

In the first group of experiments reported in this study, we have demonstrated that male mice are more susceptible to the toxic effects of difenacoum than female mice. Fall in PCA is a reliable index of the pharmacological effects of coumarin anticoagulants (i.e. a reduction in the circulating levels of factors II, VII, IX and X), but the mouse has too small a blood volume to provide serial blood samples for the measurement of PCA. Therefore, we investigated the effect of difenacoum on clotting factor synthesis in rats, and we found that after administration of the drug, PCA in male rats was both consistently, and significantly, smaller than that seen in female rats. Although it is also evident from Fig. 1 that the initial PCA of male rats was slightly lower than that seen in female rats, there was no significant difference between the two groups at this timepoint. Moreover, the differences in PCA between male and female rats at 6, 12 and 24 h were greater than those seen at t = 0. Therefore, it would appear that baseline differences do not account for our present findings. Our data are thus consistent with the suggestion that male rats and mice are more susceptible to the anticoagulant effects of difenacoum than female rats and mice.

One possible explanation of the present findings is that the plasma levels of difenacoum were lower in females than in males over the course of the experiment, due to more rapid elimination of the drug in females. However, we found that the plasma levels of difenacoum 24 h after its administration were not different in either sex.

A second explanation of our present findings is that the hepatic concentration of vitamin K may be higher in females than in males (Matschiner & Doisy 1966). In the present study, we have investigated further the possible sex-linked differences between hepatic levels of phylloquinone using HPLC with electrochemical detection. Although the mean levels of vitamin K_1 were similar in both male and female rat liver, there was a marked inter-individual variation in male rat liver that was not seen in females. At present it is unclear whether the marked differences in vitamin K_1 concentrations in male rat liver are reflected in the overall pharmacological response to anticoagulants in male rats. However, in these experiments, the pharmacological response to difenacoum in male rats was less variable than would have been predicted if hepatic concentrations of vitamin K, alone, had determined clotting times.

The concentration of phylloquinone recorded in our female rat livers (i.e. where levels of the vitamin were consistent) was less than half that seen using chick bioassay (Matschiner & Doisy 1966), but was similar to that recorded in normal male rats by Haroon & Hauschka (1983), who used HPLC with ultraviolet detection. Since HPLC methods are more specific for the determination of vitamin K_1 than is the chick bioassay, it is probable that the concentrations of vitamin K_1 seen in our experiment are a more accurate measure of the endogenous levels of the vitamin found in rat liver than the levels of vitamin K measured using chick bioassay.

Twenty-four hours after the administration of difenacoum, the levels of vitamin K_1 in both male and female rat livers were either very low, or were undetectable. This finding is consistent with the known actions of coumarin anticoagulant drugs, whereby inhibition of the vitamin K epoxide cycle blocks the normal mechanism for the conservation of endogenous vitamin K_1. In addition, we have shown that in some rats (2 out of 7 male, 6 out of 7 female rats), there was a large peak that eluted immediately before vitamin K. It is possible that the peak seen in our experiment using rat liver was the same as a peak described by Hart et al (1985) using human plasma, but it is interesting that the peak seen presently was found only in animals pretreated with difenacoum. Therefore, an alternative possibility is that our present result shows a breakdown product of phylloquinone that is seen only in the presence of coumarin anticoagulants.

In conclusion, we were able to observe a greater sensitivity of male rats and mice to difenacoum, compared with female rats and mice. Other authors

have suggested that oestrogens enhance clotting factor synthesis through a fast-acting mechanism (Rama Rao et al 1964; Owens & Cimino 1984), or over a longer timecourse (Matschiner & Willingham 1974; Siegfried et al 1979). Our present findings suggest that in addition to these possibilities, there may be a more marked rate of catabolism of vitamin K-dependent clotting factor precursors in male rats than in female rats. Whether this effect is the result of a greater turnover of plasma proteins in male rats, compared with female rats, or to the specific breakdown of vitamin K-dependent clotting factor precursors, remains to be determined.

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The effect of meptazinol on the guinea-pig sphincter of Oddi in-vitro

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Meptazinol causes a dose-dependent contraction of the guinea-pig sphincter of Oddi in-vitro. This was antagonized by atropine in concentrations which blocked the contractile response to acetylcholine but not that to KCl. Naloxone was unable to block the response of the tissue to meptazinol, and other opioid drugs had inconsistent effects. Although meptazinol has significant anticholinesterase activity on this preparation, comparison with neostigmine suggests that this is irrelevant to its contractile action.

Meptazinol is a mixed agonist antagonist opioid analgesic which has additional cholinergic analgesic activity (Bill et al 1983). Cholinergic activity is potentiated by meptazinol in smooth muscle preparations (Stephens et al 1978) and in skeletal muscle preparations (Strahan et al 1985). These actions can be explained, at least in part, by meptazinol's anticholinesterase activity (Galli 1985).

Mixed agonist antagonist opioid analgesics are reputed to be less likely to cause spasm of the sphincter of Oddi than pure agonists such as fentanyl and morphine (McCammon et al 1984). However, since the sphincter of Oddi contracts powerfully to acetylcholine (Persson 1972) it is possible that meptazinol might have greater effects on this tissue than other opioids. This

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possibility has been examined in-vitro using the guinea-pig sphincter of Oddi preparation.

Methods
Male and female tricolour guinea-pigs were killed by a blow to the head. The common bile duct, cystic duct, gall bladder and a small piece of duodenum surrounding the sphincter of Oddi were dissected out. A cannula was introduced into the common bile duct through a hole made in the gall bladder and through the cystic duct and secured so that the tip of the cannula was 5 mm above the sphincter of Oddi. The preparation was perfused through the cannula with Krebs-Henseleit solution oxygenated with 5% CO_2 in oxygen at 37 °C at 0.5 mL min^{-1} and immersed in oxygenated Krebs-Henseleit solution at 37 °C. Pressure changes in the perfusate were measured just above the tissues using a pressure transducer connected to a Grass 79C recorder. The apparatus was calibrated daily with a mercury manometer. The resting pressure of the preparations was between 5 and 25 mm Hg (mean 14 mm Hg). Drugs were applied to the tissue as a bolus in 0.1 mL of 0.9% NaCl (saline) to the inner surface of the preparation or in the perfusing Krebs solution. The outside of the