Inhibition of Cortisol Metabolism in vitro by 2-Methylnaphthoquinone (Menadione): A Possible Interpretation of the Potentiation of Cortisol Activity in vivo

Recently FEKETE and co-workers demonstrated in the rat that coadministration of menadione and corticoids, such as prednisolone or cortisol, yielded an increase in the antiphlogistic, glycogenic, and thymolytic activities of these corticoids[1-3].

The in vivo transformation of cortisol into physiologically inactive derivatives is thought to involve ring A reduction as the rate limiting step[4,5]. The rate of this reaction in vitro with rat liver microsomes depends on the concentration of the cofactor NADPH, and on the concentration of the microsomal enzymes[5,6]. The in vitro observations that menadione stimulates NADPH oxidation by microsomal systems[7-9] and that NADPH dependent metabolism of antipyrine is inhibited by menadione[9] suggested to us that the potentiation of cortisol action by menadione might involve a decrease in cortisol metabolism due to the depletion of the endogenous stores of NADPH. The present study was designed to examine this possibility[10].

Materials and methods. Albino female rats (Sherman strain) weighing 200–250 g were obtained from Wyckoff Breeding Colony, New Jersey. Liver microsomes were prepared essentially by the method of MCGUIRE and TOMKINS[11] with some modifications[12]. Prior to use, microsomes were thawed and were suspended in 0.25 *M* sucrose (1 ml of sucrose/g of original liver). Cortisol metabolism: 0.6 ml of cortisol solution (160 μg/ml of absolute methanol containing 4% v/v propylene glycol) were placed in 25 ml Erlenmeyer flasks. 0.2 ml of the solution containing menadione (dissolved in methanol containing 1% v/v propylene glycol) or an equivalent volume of the solvent were added and the resulting solutions were evaporated to dryness under N_2 (40–50 °C). The flasks were chilled in an ice bath and the following were added: 0.2 ml of the microsomal suspension, 20 μmoles *Tris* (pH 7.4), 0.49 μmoles NADPH in a total volume of 2 ml of deionized water. Aliquots (0.9 ml) were taken at zero-time and after incubation with shaking in air at 37 °C for 20 min. Each aliquot was extracted with 5 ml reagent grade methylene chloride, centrifuged to separate the phases and the absorbance in the lower (organic) phase was measured at 240 nm in a Beckman DU spectrophotometer. Loss of absorbance at 240 nm was taken to indicate reduction of ring A to yield 5α-products[11,12]. Hexobarbital metabolism: preparation of 9000 *g* liver supernatant from male rats and incubation procedures were the same as previously described[13]. The assay of residual unmetabolized hexobarbital was carried out as described by COOPER and BRODIE[14].

Results and discussion. The reduction of ring A of cortisol by female liver microsomes was inhibited by menadione (Table I). The magnitude of inhibition was lower at higher concentrations of NADPH, suggesting that the inhibition of cortisol reduction is related to interference with the availability of NADPH. To examine this further, the time course of reduction of cortisol in the presence of either estradiol or menadione was investigated. The inhibition of cortisol reduction by menadione could be partially alleviated by the addition of NADPH; however, as expected from previous observations[12,15], the inhibitory action of estradiol-17β was not diminished by NADPH (Figure). The addition of NADPH did not stimulate significantly the rate of reduction of cortisol in the absence of menadione indicating that the concentration of NADPH was not limiting in the absence of this inhibitor[16].

These findings demonstrated that menadione inhibition of ring A reduction involved an interference at the cofactor level. Such an action by menadione could have

Table I. The effect of menadione on ring A reduction of cortisol by female rat liver microsomes

Experiment No.	Menadione (*M*)	Ring A reduction (%)	Inhibition of reduction (%)
1	–	69.0	–
	10^{-4}	34.4	51
2	–	58.5	–
	10^{-5}	30.3	48
3	–	49.5	–
	10^{-4}	15.0	70
	10^{-4}	29.3[a]	41[a]

Each incubation mixture contained: 96 μg cortisol and 2 ml microsomal preparation [0.2 ml of microsomal suspension, 20 μmoles *Tris* buffer (pH 7.4), 0.49 μmoles NADPH and menadione at indicated concentrations and brought to final volume with deionized water]. 0.9 ml aliquots were taken at zero-time and after incubation at 37 °C for 20 min. Each aliquot was extracted with 5 ml methylene chloride and the organic phase was read at 240 nm in Beckman DU spectrophotometer. Preparation of microsomal suspension and experimental conditions are described in materials and methods. [a] Concentrations of NADPH was 9.6×10^{-4} *M*.

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[16] In another experiment we demonstrated that the higher absorbance in the extracts from solutions containing menadione was due to absorbance of menadione at 240 nm. There was no decrease in this absorbance when menadione was incubated under similar conditions in the absence of cortisol for 11 min. Moreover, the further addition of 1.2 μmoles of NADPH at $5^1/_2$ min of incubation did not yield a decrease in absorbance at 240 nm. These results demonstrated that the decrease in absorbance at 240 nm following NADPH addition to incubations containing both cortisol and menadione was due to an increase in reduction of ring A of cortisol and not due to changes in the absorbance of menadione.

resulted from an interference with NADPH utilization, such as competition with NADPH for the enzyme, and/or from NADPH depletion presumably by oxidation via the microsomal diaphorase system[7,8]. Evidence for the latter is provided by the observation that menadione stimulates the rate of disappearance of NADPH by liver microsomes in the absence of cortisol (Table II). Dicoumarol (bishydroxycoumarin) did not inhibit the menadione mediated stimulation of NADPH oxidation by microsomes indicating that our microsomal preparations were free of mitochondrial contamination[8,17,18]. To obtain indirect evidence for menadione action in vivo we resorted to hepatic microsomal hexobarbital oxidase as a model for an NADPH requiring system. The observed inhibition of the rate of hexobarbital metabolism in vitro by menadione (Table III) is in accordance with the concept of NADPH depletion. The duration of hexobarbital induced sleeptime is thought to be inversely related to the rate of metabolism of this barbiturate. Thus, to demonstrate in vivo NADPH depletion by menadione, the effect of menadione (25 or 50 mg/kg, given i.p.) on hexobarbital induced sleep time in adult male rats was studied. There was no prolongation of sleep time by menadione; under similar conditions the administration of β-diethylaminoethyldiphenylpropyl acetate (SKF 525A) resulted in a prolonged sleep, there was no effect by menadione on the duration of zoxazolamine sleep (unpublished results).

In conclusion, our results demonstrate that 2 NADPH requiring microsomal liver enzyme systems, cortisol-5α-Δ^4-reductase from the female rat and hexobarbital oxidase from the male rat are inhibited by menadione. The inhibition seems to result from NADPH depletion by menadione.

The lack of menadione effect on sleep might suggest that in our studies menadione does not deplete NADPH in vivo. However, these results lent themselves to other interpretations as well. Further investigation is necessary to establish whether our in vitro observations on inhibition of cortisol metabolism by menadione are a true representation of the in vivo potentiation of the pharmacological activity of cortisol[19].

Table II. Rate of depletion of NADPH by menadione in the presence of female rat liver microsomes

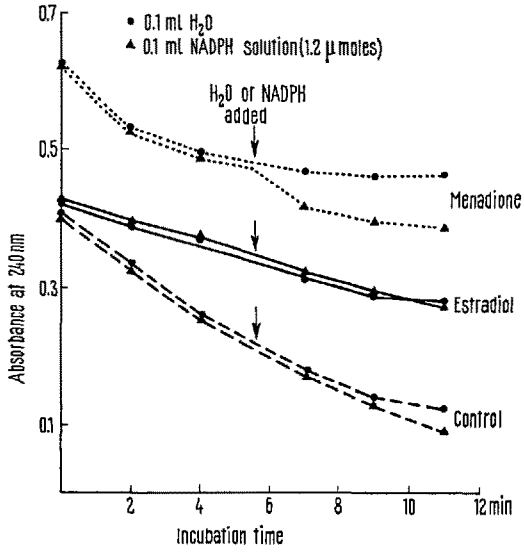
Concentration (M) Menadione	Dicoumarol	ΔO.D./min at 340 nm Experiment No. 1	2	3	4
–	–	0.005	0.004	0.0025	0.0035
1×10^{-5}	–	0.011			0.009
1×10^{-5}	1×10^{-4}				0.010
5×10^{-5}	–	0.029	0.031		
1×10^{-4}	–		0.029	0.033	
1×10^{-4}	1×10^{-4}			0.025	

Each sample contained 3.1 ml solution composed of 15 μmoles *Tris* buffer (pH 7.4), 0.15 ml of microsomal suspension, 0.36 μmoles of NADPH and menadione and dicoumarol at the indicated concentrations. Absorbances were read at 340 nm every 2 min in a Beckman DU spectrophotometer.

Table III. Effect of menadione on hexobarbital metabolism by a male rat liver supernatant[a]

Additions	Hexobarbital metabolized μg/g liver/15 min
–	427.8
Menadione ($10^{-4}M$)	172.5

Each incubation mixture contained: 1 ml of a 9000 *g* liver supernatant, 2 μmoles NADP, 40 μmoles glucose-6-phosphate, 75 μmoles $MgCl_2$, 50 μmoles nicotinamide, 1 ml 1.15% KCl, 200 μmoles phosphate buffer (pH 7.4), and 50 μg of sodium hexobarbital in a 5 ml vol. 2 ml aliquots were taken at zero-time and after incubation at 37 °C for 15 min. Preparations, conditions of incubation and assay were as described in materials and methods. [a] Sherman strain male rats weighing about 200 g were obtained from Rockland Farms.



The effect of NADPH on inhibition of cortisol reduction by menadione and estradiol-17β. Incubation mixture contained: 0.63 μmoles cortisol, 0.6 μmoles menadione or estradiol-17β (when so specified), 0.6 ml microsomal suspension (obtained from 0.6 g wet weight of liver), 2.9 μmoles NADPH, 60 μmoles phosphate buffer (pH 7.4) in a total vol. of 6 ml. $5^1/_2$ min after the start of incubation, 0.1 ml H_2O or 0.1 ml NADPH solution (1.2 μmoles) were added. $^9/_{10}$ ml aliquots were taken at 0, 2, 4, 7, 9 and 11 min, extracted with methylene chloride and their absorbance was read at 240 nm in a Beckman DU spectrophotometer. Preparations and incubation conditions are described in materials and methods.

Résumé. Le degré de réduction du noyeau A du cortisole par les microsomes du foie du rat et par le NADPH est inhibé du 50% par le méthyle-2-naphtoquinone (ménadione). L'inhibition est partiellement allégée par l'addition de NADPH ce qui indique que la ménadione gêne la disponibilité du NADPH. En effet, il s'est trouvé que la ménadione active la diminution de NADPH par les microsomes du foie. Il est possible que l'augmentation précédemment signalée de l'activité pharmacologique du cortisole par la ménadione est apparentée à l'inhibition du métabolisme du cortisole.

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