Antioxidative Properties of Phenazone Derivatives: Differentiation between Phenylbutazon and Mofebutazon

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Both phenylbutazon and mofebutazon inhibit oxidative fragmentation of the methionine derivative, 2-keto-4-methylthio-butyric acid (KMB) by xanthine oxidase − or diaphorase mediated OH radical production. Differentiation of the two non-steroidal antiinflammatory drugs is possible by means of determining oxygen reduction by xanthine oxidase or diaphorase in the presence of the naphthoquinone, juglone, where only mofebutazon shows an inhibitory effect.

Introduction

The phenazone derivatives phenylbutazon (PB) and mofebutazon (MB) are members of the NSAID-family exhibiting analgetic, antipyretic and antiinflammatory properties. While application of PB suffers from several side effects such as damage of the reticuloendothelial system, formation of edema and inhibition of blood clotting reactions [1], MB shows much less undesirable side effects. MB is structurally related with PB, containing only one benzene substituent at the pyrazole moiety thus "opening" a reactive NH-group in the heterocyclic ring system. We compared the inhibitory effects of these two NSAIDs on certain catalytic activities of XO and DIA and found that both compounds inhibit OH-radical-dependent molecule fragmentations in a similar or identical manner. However, only MB significantly influenced oxygen uptake by these enzymatic reactions and thus superoxide formation.

Materials and Methods

Diaphorase, EC 1.6.4.3 (*C. kluyveri*, 52.4 U/mg) was from Serva (Deisenhofen) and xanthine oxidase (milk, EC 1.2.3.2) was from Boehringer, Mannheim.

Abbreviations: MB, mofebutazon; PB, phenylbutazon; XO, xanthine oxidase; DIA, diaphorase; DCPIP, dichlorophenol indophenol; KMB, 2-keto-4-methylthio-butyric acid; NSAID, non-steroidal antiinflammatory drug.

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All other chemicals were purchased either from Merck (Darmstadt) or from Sigma.

Mofebutazon was a gift from Fa. Medice, Iserlohn, and phenylbutazon was obtained from Sigma.

Oxygen uptake was determined potentiometrically in 2 ml vessels with an oxygen electrode (Rank Brothers, England). The temperature of the reaction mixtures were 22, 25 and 37 °C corresponding to oxygen tensions of 532, 506 and 414 nmol O_2/2 ml, respectively.

Bleaching of DCPIP (with an assumed molar extinction coefficient of 18,200) has been measured photometrically at 601 nm, starting with an initial extinction of *ca.* 1,0.

Oxidative fragmentation of KMB was followed gaschromatographically by determining ethylene formation as described recently [2−4].

Test systems

The diaphorase system

Diaphorases and/or NAD(P)H-acceptor-oxidoreductases are ubiquitarily found as soluble or membrane-bound flavoproteins reducing oxygen monovalently in the presence of certain autooxidizable redox cofactors, such as the naturally occurring naphthoquinone, juglon (5-hydroxy-1,4-naphthoquinone, purchased from EGA-Chemie, Steinheim, Germany). The first product of this reaction in the presence of oxygen is the superoxide radical anion, $O_2^{·-}$ [3].

The test system contained in 2 ml:
100 mM phosphate buffer pH 7.4
50 μM Juglone

2.2 U diaphorase
375 mM NADH;
for measuring OH radical formation, 2.5 mM KMB were added; the reaction was conducted for 30 min at 37 °C in the dark.

The xanthine oxidase system

Xanthine oxidase oxidizes hypoxanthine, xanthine or acetaldehyde [5] under reduction of oxygen producing superoxide and hydrogen peroxide.

The test system contained in 2 ml:
100 mM phosphate buffer pH 7.4
0.5 mM xanthine
0.08 U XO
for measuring OH formation, 2.5 mM KMB were added.

The reaction was conducted for 30 min at 37 °C in the dark.

In the figures and in the table mean values of the individual experimental data are presented. These data were obtained from three to five parallels run in two independent experiments.

Results

Inhibition of XOD- and DIA-dependent KMB fragmentation

Ethylene formation from KMB by the above enzymic systems is both inhibited by catalase and superoxide dismutase indicating the cooperation of superoxide and hydrogen peroxide in this oxidative fragmentation [3]. This penomenon is best explained by a Haber-Weiss-type mechanism of OH⁻ generation [6]. As shown in Fig. 1, KMB fragmentation is almost identically influenced by MB and PB. In the XOD-catalyzed reaction (Fig. 1a), a significant inhibition starts between 0.1 and 1 mM whereas the DIA-catalyzed reaction is clearly inhibited by both MB and PB one order of magnitude lower. The uninhibited XOD-reaction produces approximately 9 to 10 nmol ethylene in 30 min and the DIA reaction produces *ca.* 13—15 nmol in the same time period.

Effects on oxygen uptake

The outlined XOD system consumes *ca.* 45 nmol O$_2$/min while the DIA reaction mixture takes up *ca.* 85 nmol O$_2$/min.
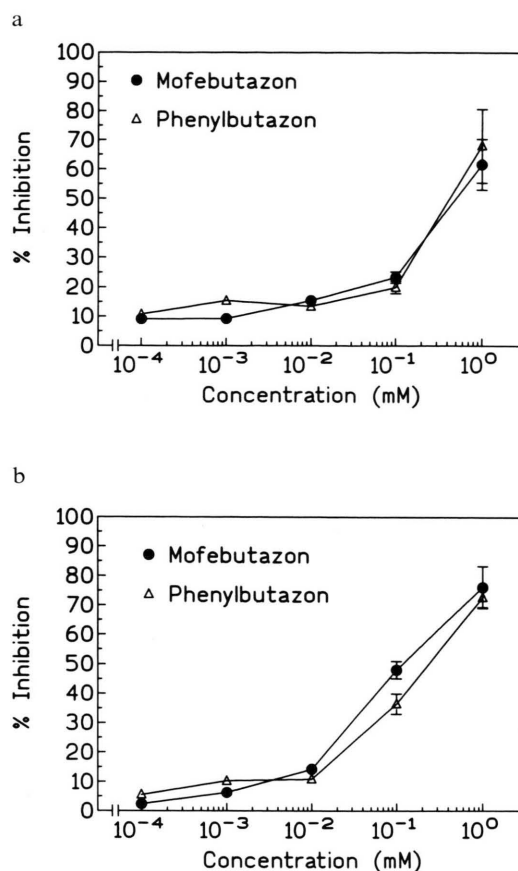

Fig. 1. Inhibition by mofebutazon and phenylbutazon of ethylene formation from KMB.
a) Xanthine oxidase-catalyzed reaction. The uninhibited reaction (0% inhibition) produced 9683 (±) 734 pmol ethylene in 30 min.
b) Diaphorase-catalyzed reaction. The uninhibited reaction produced 14142 (±) 594 pmol ethylene in 30 min.

As shown in Fig. 2, MB impairs oxygen uptake by the DIA system starting at a concentration of 0.05 mM while the same extent of inhibition by PB (*ca.* 30%) becomes only visible at a ten-fold concentration, *i.e.* 0.5 mM. At 0.5 mM MB inhibits oxygen uptake by approximately 80%.

Inhibition of oxygen uptake by the XOD system is only visible at 1.0 mM MB where only 22 nmol O$_2$/min are consumed instead of 42 nmol of the unimpaired reaction. This corresponds to a *ca.* 52% inhibition. PB has no effect on XOD-catalyzed oxygen uptake up to a concentration of 1 mM.
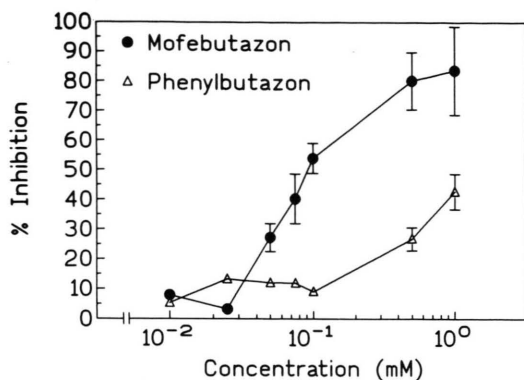
Fig. 2. Inhibition of oxygen uptake by mofebutazon during the diaphorase- and juglon-catalyzed NADH oxidation. Two independent sets of reactions were conducted: At 0% inhibition, oxygen uptake in the mofebutazon experiment was 83.9 ($\pm$) 7.8 nmol O_2/min, and in the phenylbutazon experiment 90.1 ($\pm$) 11.9 nmol O_2/min.

Effects on DIA-catalyzed DCPIP reduction

In contrast to oxygen reduction, DCPIP reduction by DIA at the expense of NADH as electron donor is not dependent on the addition of juglone and represents a two electron step directly between the isoalloxazin moiety of the reduced enzyme and the non-autooxidizable electron acceptor, DCPIP. As shown in Table I both MB and PB exhibit an approximately 16 to 17% inhibition of DCPIP re-

Table I. Effects of mofebutazon and phenylbutazon on DIA-catalyzed DCPIP reduction.

Concentration [mM]	% reaction MB	PB
0.01	105	99
0.1	102	98
1.0	85	83

Standard deviations: between ca. 9 to 13%.

duction at 1 mM whereas lower concentrations are without effect.

Discussion

The obtained results may be interpreted as follows:

Besides the functions known for NSAIDs, *i.e.* inhibition of enzymic reactions involved in the inflammatory reaction chain (*i.e.* steps of the arachidonic acid cascade) finally yielding hormone – like mediators such as prostaglandins and leucotriens, MB and PB act as inhibitors of OH radical – mediated destructions, shown as decrease of XOD- or DIA-catalyzed KMB fragmentation. This type of inhibition holds for broad variety of substances [3, 6] since the OH-radical is extremely reactive. Competition with oxygen involved in the one electron transfer, however is only obtained in the case of MB. This may be explained by the fact that only MB possesses a reactive NH-group in the heterocycle. In the OH-driven reaction (detected at a molar platform approximately one order of magnitude below the one for oxygen reduction) the transition metal- or semiquinone-dependent hydrogen peroxide reduction and not the oxygen reduction is the limiting step [7]. Thus, MB only competes with oxygen in the one electron step at the negative (E_0' for $O_2/O_2^{·-}$ = -0.33 V) potential whereas at the extremely positive potential of the OH-type reaction (E_0' = *ca.* $+2$ V) no difference between the two NSAIDs is observed. The fact that neither MB nor PB interact dramatically with the flavin-type two-electron transfer is shown by the lack of significant inhibition of the DIA-catalyzed DCPIP reduction. Therefore, MB in contrast to PB may prevent excess superoxide and thus also hydrogen peroxide formation during inflammatory processes and thus positively influence its therapeutic effect, avoiding undesirable side effects.

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