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Relationship between the rate of reduction of benzo(*a*)pyrene-3,6-quinone and the formation of benzo(*a*)pyrene-3,6-quinol glucuronides in rat liver microsomes

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A major class of metabolites formed during microsomal monooxygenation of benzo(*a*)pyrene (BP)* are the BP-quinones [1–3]. These products have been shown to inhibit the mixed function oxidation of BP [4] which probably is due to substrate competition since BP-3,6-quinone is actively "recycled" through the cytochrome P-448 linked monooxygenase system [5]. This activity, however, is drastically decreased when BP-3,6-quinone is metabolized to its glucuronosyl conjugates [5] and could thus explain the observed increase in the total metabolism of BP in the presence of UDP-glucuronic acid (UDPGA) [4–7]. *In vivo* glucuronidation of BP quinones appears to be an important pathway since BP-quinol glucuronides constitute one of the major groups of conjugates found in the bile of rats during metabolism of BP [8].

It has been reported [5] that reduction of BP-3,6-quinone is a prerequisite for the glucuronidation of BP-3,6-quinone. Both DT diaphorase (NAD(P)H:quinone oxidoreductase) and NADPH-cytochrome P-450 reductase is known to catalyse this reaction [5, 7, 9]. However, DT diaphorase is a two-electron transfer enzyme [10] and as such will give rise to hydroquinones. In contrast when any quinone is reduced by a one-electron transfer enzyme such as NADPH-cytochrome P-450 reductase, a semiquinone will always be formed [10]. Semiquinones are in general very labile and are readily autooxidized with the formation of O_2^- and H_2O_2. This basic difference between the two enzymes has been demonstrated in liver microsomes with the quinone menadione [11]. The ratio between hydro- and semiquinone formation was shown to be dependent on which pathway was prevailing, e.g. in the presence of selective inhibitors of the enzymes or by treatment of the rats with phenobarbital (PB) or 3-methylcholanthrene (MC) which preferentially induces NADPH-cytochrome P-450 reductase and DT diaphorase, respectively.

In previous reports on the rate of reduction of BP-3,6-quinone in microsomes [9] and hepatocytes [12] it was suggested that NADPH-cytochrome P-450 reductase is the major BP-3,6-quinone reductase in the formation of BP-3,6-quinol glucuronides. Although no measurement of glucuronide formation was performed a role for DT diaphorase as a BP-3,6-quinone reductase in this metabolic pathway was ruled out [9, 12]. The suggestion was based on results such as lack of NADH-supported reduction of BP-3,6-quinone [9] and that the rate of reduction appeared to be much faster via NADPH-cytochrome P-450 reductase relative to DT diaphorase [9, 12]. Lorentzen and Ts'o have reported [13] that not only the semiquinones of BP-3,6-quinone but also the hydroquinones are easily oxidized by oxygen with a concomitant formation of O_2^- and H_2O_2. Thus unless the rate of reduction of BP-3,6-quinone and the rate of subsequent conjugation reaction is comparable, the reduction of BP-3,6-quinone, especially via NADPH-cytochrome P-450 reductase, will be a very cytotoxic reaction pathway. The present study was undertaken in order to establish the relationship between the reduction of BP-3,6-quinone and the formation of glucuronides in liver microsomes from both PB- and MC-treated rats.

* Abbreviations used: BP, benzo(*a*)pyrene; PB, phenobarbital; MC, 3-methylcholanthrene; UDPGA, UDP-glucuronic acid.

Materials and methods

BP-3,6-quinone was a generous gift from Dr. M. Litwack, IIT Research Institute (Chicago, IL., U.S.A.). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO.) except for MC which were purchased from Fluka AG (Buchs, Switzerland).

Microsomes were isolated from MC- and PB-treated male Sprague–Dawley rats (100 g) as described in [11, 14].

Reduction of BP-3,6-quinone (10 μM, dissolved in hexamethylphosphoramide) in microsomes from PB- or MC-treated rats was assayed as described by Capdevila *et al.* [9] in the presence of 50 mM Tris–Cl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM NADPH or NADH and 2 mM UDPGA.

The formation of BP-3,6-quinol glucuronides was assayed essentially as described by Bock *et al.* [15]. Aliquots of 0.1 ml were withdrawn from the cuvettes during the assay of BP-3,6-quinone reduction and the reaction was stopped by the addition of 0.2 ml ice cold methanol. Precipitated protein was discarded and 1 ml 0.3 M glycine buffer, pH 10.3, was added to the samples. The emission spectrum of the samples was recorded in an Aminco-Bowman spectrofluorometer between 350 and 650 nm employing an excitation wavelength of 400 nm.

All the results are representative of at least three different determinations performed at room temperature.

Results and discussion

Spectral analysis of the reduction of BP-3,6-quinone in microsomes from PB-treated rats in the presence of NADPH has been reported [9] to result in a decrease in the absorption of the quinone at 480 nm with a concomitant formation of two peaks at 401 and 448 nm, identical to those obtained with the reduced quinone. In the present investigation a decrease in the absorption at 480 nm is also observed when BP-3,6-quinone is metabolized in the presence of NADPH and UDPGA in microsomes from either PB-treated (Fig. 1A, trace 3) or MC-treated rats (Fig. 1B, trace 3). With time though the peaks at 401 and 448 nm are shifted to lower wavelengths. A similar shift of the peaks has previously been observed in hepatocytes by Capdevila and Orrenius [12], who suggested these two spectral species to be correlated to the formation of conjugates. However, in microsomes from MC-treated rats the shift of the peak at 448 nm is much more pronounced (Fig. 1B, trace 3), more than 20 nm after 20 min incubation (not shown) than in microsomes from PB-treated rats indicating a more complex picture of the formation of BP-3,6-quinol glucuronides (see below).

Since NADH and NADPH is known to be equally good as hydrogen donors for DT diaphorase [16] the metabolism of BP-3,6-quinone was investigated in microsomes from either PB- or MC-treated rats in the presence of UDPGA and NADH rather than NADPH. Spectral analysis revealed that NADH indeed could serve as hydrogen donor in the reduction of BP-3,6-quinone (Fig. 1, trace 4). The rate of reduction of BP-3,6-quinone though, i.e. the decrease in absorption at 480 nm, was much slower relative to that observed with NADPH (Fig. 1A, trace 3) in microsomes from PB-treated rats (where NADPH-cytochrome P-450 reductase is more abundant than DT diaphorase [11]. In microsomes from MC-treated rats (where the relative activity of the two enzymes is opposite to that in microsomes

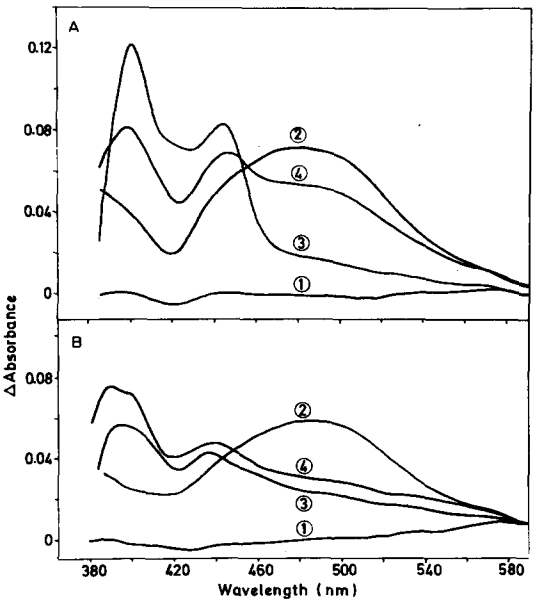

Fig. 1. Absorption spectrum of BP-3,6-quinone in microsomes from (A) PB or (B) MC-treated rats in the presence of UDPGA. A suspension of microsomes from (A) PB- (0.8 mg/ml) or (B) MC-treated rats (0.6 mg/ml) containing 2 mM UDPGA was placed in the sample and reference cuvette and a baseline (1) was recorded. After addition of 10 μM BP-3,6-quinone to the sample cuvette a spectrum was recorded (2). The reaction was initiated by the addition of either NADPH (0.5 mM, 3) or NADH (0.5 mM, 4) to the sample and reference cuvette and a spectrum was recorded after 4 min (3 and 4, respectively).

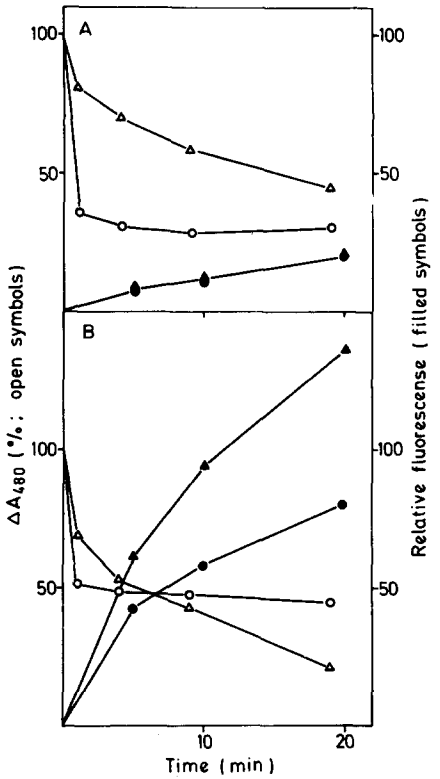

Fig. 2. Relationship between the reduction of BP-3,6-quinone and the formation of BP-3,6-quinol glucuronides in microsomes from (A) PB- or (B) MC-treated rats. The reduction of BP-3,6-quinone, indicated by the decrease in absorption at 480 nm, in the presence of UDPGA (2 mM) and either NADPH (0.5 mM, ○—○) or NADH (0.5 mM, △—△) was followed as specified in Fig. 1. After recording a difference spectrum, at times indicated, an aliquot of 0.1 ml was withdrawn from the sample cuvette and the reaction was stopped by the addition of methanol as described under Materials and Methods. The fluorescence of the BP-3,6-quinol glucuronides formed in the presence of NADPH (●—●) or NADH (▲—▲) was measured as specified under Materials and Methods.

from PB-treated rats [11]) this difference between the hydrogen donors was less pronounced (Fig. 1B). Capdevila *et al.* [9] have previously reported that NADH cannot support the reduction of BP-3,6-quinone in microsomes from PB-treated rats. However, these authors monitored the reduction of BP-3,6-quinone in the dual wavelength mode (480–458 nm) and their results could be due to the fact that no true isobestic point of the oxidized and reduced quinone is found in the presence of NADH.

So far, many of the reports concerning formation of BP-3,6-quinol glucuronides have been focused on the first reaction, i.e. the reduction of BP-3,6-quinone [9, 12]. In the present investigation the correlation between quinone reduction and the formation of BP-3,6-quinol glucuronides has been studied. Thus, the metabolism of BP-3,6-quinone in microsomes from either PB- or MC-treated rats in the presence of UDPGA and NAD(P)H was followed by recording the absorption spectrum between 360 and 600 nm at different times taking the decrease in absorbance at 480 nm as a measure of BP-3,6-quinone reduction. Aliquots were withdrawn from the cuvette at times indicated in Fig. 2 and analysed for the content of BP-3,6-quinol glucuronides with a fluorescent method described by Bock *et al.* [15]. In microsomes from PB-treated rats, where the rate of reduction of BP-3,6-quinone was much faster with NADPH relative to NADH (Fig. 1A, traces 3 and 4 respectively, Fig. 2A), the same amount of glucuronides was found irrespective of hydrogen donor used (Fig. 2A). Furthermore, in microsomes from MC-treated rats, where the rate of reduction of BP-3,6-quinone was much slower than in microsomes from PB-treated rats (Fig. 2B and 2A) the amount of glucuronides formed (Fig. 2B) was significantly higher relative to that in microsomes from PB-treated rats (Fig. 2A). These results show that no direct correlation exists between the rate of reduction of BP-3,6-quinone and the formation of BP-3,6-quinol glucuronides and that the reduction of BP-3,6-quinone is probably not the rate-limiting step in glucuronide formation. Consequently the rate by which an enzyme catalyses the reduction of BP-3,6-quinone is not *a priori* related to its role in the formation of the glucuronosyl conjugates. Thus, though part of the semiquinones formed by NADPH-cytochrome P-450 reductase will dismutate to hydroquinones [9–10] one cannot exclude the possibility that DT diaphorase, which exclusively forms hydroquinones, plays a significant role also in the formation of BP-3,6-quinol glucuronides. This assumption is also supported by the present finding that NADH can replace NADPH as the hydrogen donor thus the enzyme could very well be DT diaphorase. Furthermore, it has previously been reported [5] that the amount of BP-3,6-quinol glucuronides formed in microsomes devoid of NADPH-cytochrome P-450 reductase was very similar to that found in intact microsomes [5].

The results presented in Fig. 2 also reveal that in microsomes from MC-treated rats the amount of BP-3,6-quinol glucuronides formed is much higher in the presence of NADH relative to NADPH. These results could be an additional support for the above mentioned role of DT

diaphorase in the formation of BP-3,6-quinol glucuronides. However, it has been shown [5] that in microsomes from MC-treated rats BP-3,6-quinone is actively "recycled" via the cytochrome P-448 linked monooxygenase system to products no longer suitable as substrates for UDP-glucuronosyltransferase. Thus, the difference between the NADH- and NADPH-linked conjugation reaction might at least partly be explained by a lower concentration of BP-3,6-quinone available in the latter reaction.

As mentioned above the differences in the absorption spectra of metabolized BP-3,6-quinone observed in microsomes from PB-treated rats (Fig. 1A) relative to microsomes from MC-treated (Fig. 1B) in the presence of NAD(P)H and UDPGA could be an indication of a complexity in the formation of BP-3,6-quinol glucuronides. During the course of this investigation results have been obtained confirming this possibility. Thus in microsomes from MC-treated rats metabolites of BP-3,6-quinone are formed which exhibit an emission spectrum (Fig. 3), identical to that reported for BP-3,6-quinol glucuronides by Bock *et al.* [15]. However, when BP-3,6-quinone is metabolized in microsomes from PB-treated rats in the presence of NAD(P)H and UDPGA a different emission spectrum of the products is observed (Fig. 3). In addition to the double peak at 440, 460 nm, an emission maximum at 530 nm is also found. After treatment of the samples with β-glucuronidase both emission peaks disappear suggesting the existence of two different glucuronides of BP-3,6-quinol. The identity of these glucuronides, a mono- and a diglucuronide (corresponding to the 530 nm and 440, 460 nm species respectively) have been investigated as well as the possible reaction sequences by which they are formed and will be presented elsewhere [17].

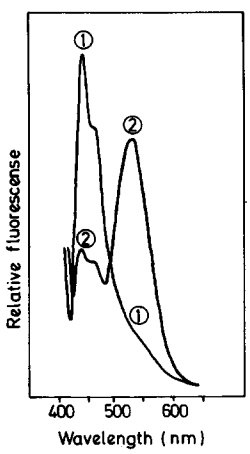

Fig. 3. Emission spectra of BP-3,6-quinol glucuronides formed in microsomes from MC-(1) or PB(2)-treated rats. Aliquots, 0.1 ml, from the content of the sample cuvette (as specified in Fig. 1) containing microsomes from either MC(1) or PB(2) treated rats were treated as described under Materials and Methods. The fluorescence of the BP-3,6-quinol glucuronides, formed after 20 min incubation, was recorded between the emission wavelengths 350–650 nm employing an excitation wavelength of 400 nm.

The present investigation shows that no direct correlation exists between the rate of reduction and the formation of BP-3,6-quinol glucuronides and that the reduction of BP-3,6-quinone is not the rate-limiting step in glucuronide formation. Furthermore, replacing NADPH with NADH as hydrogen donor reveals that the same or even higher amounts of BP-3,6-quinol glucuronides are formed in liver microsomes from both PB- and MC-treated rats. Thus, it appears likely that DT diaphorase can play a significant role as BP-3,6-quinone reductase in this metabolic pathway which results in the formation of both mono- and diglucuronides of BP-3,6-quinol.

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