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AFLATOXIN INHIBITION OF AVIAN HEPATIC MITOCHONDRIA

ONYECHI OBIDOA and H. TAUFIQ SIDDIQUI
Department of Biochemistry, Ahmadu Bello University, Zaria, Nigeria

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Abstract—Aflatoxin B_1 (AFB_1) inhibited the respiration of isolated guinea fowl (*Numida meleagris* Pearl) liver mitochondria measured by polarographic methods. The degree and site(s) of inhibition depended on aflatoxin concentration, the presence or absence of ADP and the respiratory substrate used, but in all cases was not relieved by 2,4-dinitrophenol. The major sites of inhibition of electron transport are probably localized at the first and second phosphorylation coupling sites.

The aflatoxins are environmental food contaminants in many parts of the world and are potent hepatocarcinogens in many animal species and possibly man. The absence of any satisfactory explanation for their mode of induction of toxic and carcinogenic lesions continues to motivate investigations along these lines [1].

Aflatoxin is reported to induce decreases in certain mitochondrial dehydrogenases and electron transfer catalysts in ducklings and chickens [2, 3]. These results have been interpreted as indicating a transient aflatoxin-induced disturbance of the redox mechanism of the electron transport system in avian species [4]. Germane to these experiments is the question whether the necrotic and carcinogenic lesions generally associated with aflatoxin pathology in birds could be related to aflatoxin inhibition or uncoupling of avian hepatic mitochondrial respiratory functions.

In this paper, a more detailed examination of the effect of aflatoxin B (AFB_1), the most potent aflatoxin, on electron transport and oxidative phosphorylation in the guinea fowl (*Numida meleagris* Pearl) hepatic mitochondria is presented.

MATERIALS AND METHODS

AFB_1 (Makor chemical product, Israel) solutions of graded concentrations were prepared in dimethylsulfoxide (DMSO).

Preparation of mitochondria. Mitochondria were isolated from the livers of adult male guinea fowls weighing about 1.5 kg. The birds were slaughtered by decapitation and profuse bleeding was encouraged with running tap water. The livers were quickly excised and immediately placed in ice-cold 0.25 M isotonic sucrose solution, pH 7.0. The weighed livers were homogenized and mitochondria isolated and suspended in 0.25 M sucrose such that 1 ml of suspension contained approximately 10 mg protein [5]. All experiments with mitochondria were completed within 5 hr of slaughtering the birds. Protein was estimated by the method of Lowry *et al.* [6].

Measurement of oxygen consumption. Oxygen consumption was measured polarographically with an OX1-39 oxygen meter (Jurgens & Co., Germany), employing a Clark electrode. The electrode was housed in a 3-ml reaction vessel which was enclosed in a water jacket and water circulated at 30°. Calibration of the oxygen meter was accomplished using a 3% (w/v) solution of sodium sulfite as the zero standard and aerated 0.1 M potassium phosphate buffer, pH 7.4, as the saturated standard solution.

The oxygen electrode solution for the mitochondrial preparation was a modification of that of Estabrook [7]. The final volume was 3.0 ml and it contained 25 mM sucrose, 7 mM potassium phosphate, 0.1 mM magnesium chloride, 25 mM potassium phosphate buffer, pH 7.4, 16.6 mM substrate, AFB_1 solution or DMSO in the case of control reactions. DMSO does not affect mitochondrial respiratory functions [8]. To each reaction system was added the mitochondrial suspension containing approximately 1 mg protein. When the respiratory substrate was α-ketoglutarate, 6.6 μM NAD^+ was also added, and with ascorbate as substrate 6.6 μM tetramethyl *p*-phenylene diamine (TMPD) was added.

When a steady rate of oxygen consumption was observed, 1.3 mM ADP was added to induce state 3 respiration for reactions in which α-ketoglutarate and succinate were the substrates.

Additional series of traces were run in which 0.1 mM of 2,4-dinitrophenol (DNP) was incorporated in the reaction media to observe the effect of 3.3×10^{-6} M AFB_1 in the presence of an uncoupler.

ADP:O ratio. This was determined polarographically from the oxygen uptake traces of the various reactions [7].

RESULTS

Effect of aflatoxin B_1 on electron transport. Addition of different concentrations of AFB_1 to guinea fowl liver mitochondria actively respiring on various substrates resulted in inhibition of oxygen consumption. This represents the results of a minimum of two different preparations of mitochondria with the experiments done in duplicate. The maximum concentration of AFB_1 employed was 3.3×10^{-6} M. Inhibition of oxygen consumption decreased with a decreasing concentration of AFB_1.

Figure 1 shows AFB_1 inhibition of the oxidation of α-ketoglutarate, an NAD-dependent substrate. A

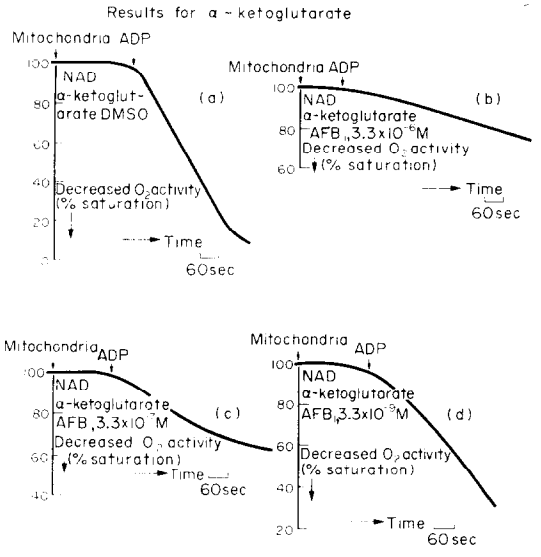

Fig. 1. Effects of different concentrations of aflatoxin B_1 (AFB_1) on the consumption of oxygen by mitochondria pulsed with ADP (1.3 mM), while metabolizing α-ketoglutarate. Basal reaction medium is presented in the text. Shown are oxygen traces in the presence of AFB_1 at concentrations of: (a) 0 (100 μl DMSO); (b) 3.3×10^{-6} M; (c) 3.3×10^{-7} M; and (d) 3.3×10^{-9} M.

maximum inhibition of about 80 per cent was observed at an AFB_1 concentration of 3.3×10^{-6} M. Figure 2(a) represents the pattern of inhibition of the oxidation of this substrate as a function of AFB_1 concentration.

A complete interruption of oxygen uptake was observed when succinate was the respiratory substrate at an AFB_1 concentration of 3.3×10^{-6} M (Fig. 3).

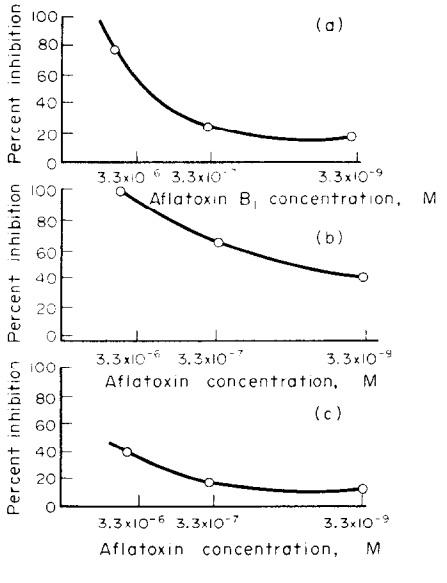

Fig. 2. Inhibition of oxygen consumption by mitochondria respiring on different substrates as a function of aflatoxin B_1 concentration. Substrates used were: (a) α-ketoglutarate; (b) succinate; and (c) ascorbate plus TMPD.

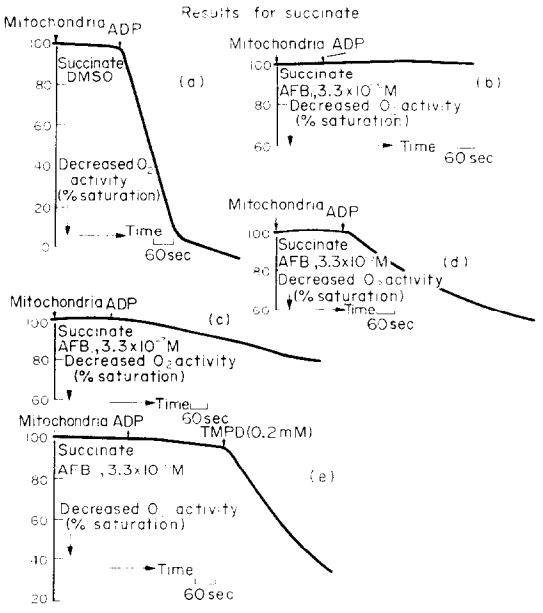

Fig. 3. Effects of different concentrations of aflatoxin B_1 (AFB_1) on the consumption of oxygen by mitochondria pulsed with ADP (1.3 mM), while metabolizing succinate. Basal reaction medium is presented in the text. Shown are oxygen traces in the presence of AFB_1 at concentrations of: (a) 0 (100 μl DMSO); (b) 3.3×10^{-6} M; (c) 3.3×10^{-7} M; (d) 3.3×10^{-9} M; and (e) 3.3×10^{-6} M and pulsed with TMPD (6.6 μM).

Succinate is an FAD-dependent substrate and donates its electrons specifically to coenzyme Q and thus bypasses the first phosphorylation coupling site in its oxidation pathway. Figure 3(e) shows that, although succinate oxidation is inhibited by AFB_1, the major point of inhibition cannot be at the dehydrogenase site, since TMPD significantly overcomes the effect. TMPD is known to shunt electrons between cytochrome *b* and cytochrome *c* (or C_1), thereby forestalling phosphorylation at the second coupling site [9]. This suggests that the possible site of action of AFB_1 on the respiratory chain is at the second crossover point.

Figure 4 shows the effects of AFB_1 on the oxidation of ascorbate. The process was inhibited slightly, and the maximum inhibition observed with 3.3×10^{-6} M AFB_1 was about 40 per cent of the aflatoxin-free control. The non-enzymatic transfer of electrons from ascorbate to TMPD and the subsequent oxidation of the reduced TMPD are mediated by cytochrome *c* oxidase and are coupled to the third phosphorylation site. This coupling site, therefore, was partially inhibited at the highest level of AFB_1 tested.

Effect of aflatoxin B_1 on oxidative phosphorylation. Table 1 shows the effect of different concentrations of AFB_1 on the ADP:O ratio. Using a selection of substrates, an examination of each phosphorylation site is shown. The ADP:O ratio data indicate that AFB_1 did not affect phosphorylation at the third site but did at the first and second sites, denoting the inhibition of the electron transport at these sites. The data illustrate the bypass of the AFB_1-sensitive site of the respiratory chain by means of TMPD, while

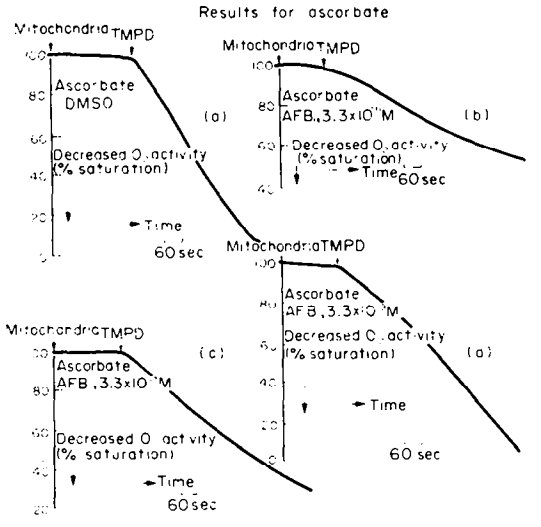

Fig. 4. Effects of different concentrations of aflatoxin B_1 (AFB_1) on the consumption of oxygen by mitochondria metabolizing ascorbate and pulsed with TMPD (6.6 μM). Basal reaction medium is presented in the text. Shown are oxygen traces in the presence of AFB_1 at concentrations of: (a) 0 (100 μl DMSO); (b) 3.3×10^{-6} M; (c) 3.3×10^{-7} M; and (d) 3.3×10^{-9} M.

lowering the P:O ratio by approximately one-third with α-ketoglutarate and about one-half with succinate as substrates. The results are consistent with the conclusion that the TMPD shunt circumvents coupling site II of the respiratory chain. When AFB_1 was present, only the shunt was operating and the P:O ratio was lowered by one unit. When AFB_1 was absent, respiration proceeded by both the normal pathway and the TMPD shunt, which resulted in an increased oxygen consumption and, therefore, decreased the P:O ratio. With ascorbate plus TMPD, respiration was unexpectedly insensitive to AFB_1, as shown by the same value for the P:O ratio obtained.

Table 2 shows the data obtained when DNP was used to stimulate oxygen uptake and indicates that

Table 1. Effect of AFB_1 on the ADP:O (P:O) ratio of isolated guinea fowl liver mitochondria*

AFB_1 concn (M)	ADP:O (P:O) ratios		
	α-Ketoglutarate TMPD	Succinate TMPD	Ascorbate plus TMPD
0 (DMSO)	2.55	1.85	0.97
3.3×10^{-6}	1.65	0.98	0.97
3.3×10^{-7}	1.72	1.02	0.98
3.3×10^{-9}	1.91	1.16	0.97

* Basal reaction medium contained, in 3 ml, 25 mM sucrose, 7 mM potassium phosphate, 0.1 mM magnesium chloride, 25 mM potassium phosphate buffer, pH 7.4, 16.6 mM substrate, 6.6 μM TMPD, 1.3 mM ADP, 6.6 μM NAD^+ when α-ketoglutarate was the substrate, and about 1 mg of mitochondrial protein. Aflatoxin additions were made as indicated on the table.

Table 2. Inhibition of oxygen consumption by AFB_1 in the presence of DNP*

AFB_1 concn (M)	Percentage inhibition with AFB_1		
	α-Ketoglutarate	Succinate	Ascorbate
0 (DMSO)			
3.3×10^{-6}	76.1	93.2	44.7

* Basal reaction medium contained, in 3 ml, 25 mM sucrose, 7 mM potassium phosphate, 0.1 mM magnesium chloride, 25 mM potassium phosphate buffer, pH 7.4, 16.6 mM substrate, 0.1 mM DNP and about 1 mg of mitochondrial protein. NAD^+ (6.6 μM) was also added when α-ketoglutarate was used as substrate. Aflatoxin addition was made as indicated on the table.

AFB_1 inhibited oxygen uptake by acting on the chain itself. Again maximum inhibition was observed with succinate as substrate.

DISCUSSION

The inhibition of rat liver mitochondrial electron transport by AFB_1 has been reported [8, 10, 11] and also that the major site of inhibition is between cytochrome *b* and *c* (or C_1). Only one report states that it inhibits specifically the succinic dehydrogenase [12].

This study represents the first collaborative attempt to verify such reported effects *in vitro* of aflatoxin B_1 on mitochondrial respiration in another vertebrate class of animals susceptible to aflatoxin hepatotoxicity, liver cell necrosis and carcinogenesis.

The results of the present study are not totally in agreement with the inhibition *in vitro* of electron transport by AFB_1 previously reported [8, 10, 11]. Doherty and Campbell [10, 11] reported an inhibition of respiration by 25–44 per cent when 2.5 to 4.8×10^{-4} M AFB_1 was added to actively respiring mitochondria, while another group [8] reported inhibition of state 3 respiration by about 50–75 per cent in the presence of AFB_1 at concentrations of 1.0 to 3.0×10^{-4} M. In our study with guinea fowl liver mitochondria, state 3 oxidation of NAD- and FAD-dependent substrates was inhibited by about 80 and 100 per cent respectively in the presence of AFB_1 at a concentration of 3.3×10^{-6} M.

It is significant that the concentration of AFB_1 which induced a higher percentage inhibition in the respiration of guinea fowl liver mitochondria is about 100 times lower than that reported for the rat. The major site of inhibition is probably indicated by the fact that TMPD overcomes the inhibition of succinate oxidation significantly. The lower level of inhibition (40 per cent) observed when ascorbate (plus TMPD) was the substrate and in the absence of ADP may be explained by the observation [8] that inhibition caused by AFB_1 is at least partly reversible with ADP. This is substantiated by the data (Table 1) on the effect of AFB_1 on the ADP:O (P:O) ratio with ascorbate (plus TMPD) as substrate in which no change in value was observed. Thus, under the mitochondrial respiratory state 4 (i.e. the controlled or resting state), AFB_1 may be capable of inhibiting respiration at the cytochrome oxidase (site III) level

in addition to coupling site II (between cytochrome *b* and *c* (or C_1). Furthermore, the maximum inhibition (100 per cent) observed with succinate, in spite of the known great avidity of mitochondria for this substrate and its preferential oxidation even in the presence of other substrates, suggests that AFB_1 is also capable of inhibiting respiration at the succinic dehydrogenase level. This is probably substantiated by the observation that at the same AFB_1 concentration (3.3×10^{-6} M) the oxidation of α-ketoglutarate is inhibited by only 80 per cent. Studies on the effect of AFB_1 on mitochondrial dehydrogenases of the guinea fowl liver revealed that α-ketoglutarate and succinate dehydrogenases were inhibited by about 80 and 4 per cent, respectively, at the concentration of 3.3×10^{-6} M [13].

It is also reported that aflatoxin B_1 uncouples oxidative phosphorylation [8] at the concentration of 1×10^{-6} M. Our results do not confirm this effect with guinea fowl liver mitochondria. In contrast, even at the lower level of AFB_1 concentration (3.3×10^{-9} M), inhibition (10–40 per cent) was observed with all the substrates tested.

It is reasonable to suggest that any differences in the effect of AFB_1 on guinea fowl liver mitochondrial respiration and that of the rat liver may be attributed to inherent species differences, since this study reveals that AFB_1 inhibition of guinea fowl liver mitochondrial respiration is not localized at coupling site II but may also involve inhibition around site I. Inhibition may also occur at the cytochrome oxidase level depending on the rate of respiration. These findings probably explain the greater susceptibility of the avian species to aflatoxin toxicity.

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