Effects of Scopoletin and Aflatoxin B_1 on Bovine Hepatic Mitochondrial Respitatory Complexes, 2: *a*-Ketoglutarate Cytochrome *c* and Succinate Cytochrome *c* Reductases

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Z. Naturforsch. **56 c,** 278–282 (2001); received August 10/October 27, 2000

Scopoletin, Aflatoxin B_1, Mitochondrial, Cytochrome *c* Reductases

The *in vitro* effects of the toxin coumarin compounds scopoletin and aflatoxin B_1 (AFB_1) on bovine (*Bos indicus*) hepatic mitochondrial respiratory complex III enzymes, succinate cytochrome *c* and *a*-ketoglutarate cytochrome *c* reductases, were examined. Kinetic studies on the interaction of the toxins with the enzymes were also carried out. The results showed that although the observed inhibitory and stimulatory effects of the two toxins were consistent with the changes in the kinetic parameters (K_m and V_{max} values), these parameters were not consistent with the observed effects of the toxins at certain concentrations. These observations are discussed in terms of the relative locations of the enzymes in the mitochondria, and the previously reported inhibitory and uncoupling effects of the toxins on cow liver mitochondrial respiration.

Introduction

The alteration of cellular metabolic processes are very important indices in the aetiology of chronic degenerative disease processes in man and animal systems (Boyd *et al.*, 1982) In both carcinogenic and non-carcinogenic disorders of the liver, the underlying biochemical lesion is the derangement (involving either inhibition or activation) of one or more essential metabolic processes of orgenelles in the hepatocyte. Mitochondrial damage is believed to be one of the primary events in the genesis of hepatocyte damage (Kulkarni and Hudgson, 1980). Such damages could be elicited by a number of xenobiotics (such as the aflatoxins) present in foods (WHO 1979).

The coumarin compounds aflatoxin B_1 (AFB_1) and scopoletin are known food contaminants (Sargeant *et al.,* 1961; Minamikawa *et al.,* 1967). They could be retained in human body when taken through diet (Hendrickse *et al.,* 1983; Obasi and Obidoa, 1995).

In particular, traces of AFB_1 have been detected in the liver and muscle samples of cows slaughtered in our locality (unpublished laboratory data). Both AFB_1 and scopoletin could be ingested by the bovine species through diet (Uzoho, 1991) hence the curiousity as to the possible effects of both compounds in the bovine species.

In our previous studies, we showed that both AFB_1 and scopoletin interfered with bovine hepatic mitchondrial respiration *in vitro* (Obasi and Obidoa, 1997a). Interference with mitochondrial respiration could imply that the compounds interacted with a number of the enzymatic processes of respiration, particularly the respiratory complexes. Earlier, we have described the effects of both scopoletin and AFB_1 on cow liver mitochondrial *a*-ketoglutarate dehydrogenase (complex 1) and succinate dehydrogenase (Complex II) (Obasi and Obidoa, 1994). We are progressing with the systematic investigations of the *in vitro* effects of these two compounds on the components of the bovine hepatic mitochondrial respiratory chain, with a view to understand the pattern of the effects of the compounds in an animal like the bovine species. In the present study, we have compared the effects of AFB_1 and scopoletin on the bovine hepatic mitochondrial respiratory complex III enzymes, the *a*-ketoglutarate cytochrome *c* and succinate cytochrome *c* reductases.

Materials and Methods

Chemical and reagents

The common reagents and chemicals used in these experiments were of analytical grade, the

0939–5075/2001/0300–0278 $ 06.00

highest purity available, and obtained from Sigma Co (St. Louis, USA), BDH (Poole, England) or Merck (Darmstadt, Germany).

The purity of both scopoletin (Serva Fein-Biochemica, Heidelberg) and AFB_1 (Sigma) was in each case, determined by the production of a single bluish fluorescent (UV-short wavelength) spot when the compound was subjected to thin-layer chromatography using methanol in chloroform (3: 97 v/v) as the development solvent (Obidoa and Obasi, 1991).

Isolation of bovine hepatic mitochondria

Liver samples (approximately 20 g) were freshly obtained from male cow (*Bos indicus*) slaughtered at the local abattoir. The liver samples (collected within 10 min after decapitating the animals) were placed in ice-cold 0.34 M sucrose. After peeling off the outer coat of the liver (liver pulp), weighed fractions of the samples were minced and washed with 0.34 M sucrose solution. Mitochondria were subsequently isolated from washed liver slices following the procedures described by Johnson and Lardy (1967) using 0.34 M sucrose as isolation medium. Isolated mitochondria were re-suspended in 0.34 M sucrose – 0.02 M Tris (hydroxymethyl) aminomethane-HCl buffer (pH 7.4). Mitochondrial protein was determined by the Biuret method (Thorne, 1984).

Measurement of the enzyme activities

The liver mitochondrial cytochrome *c* reductase activities were assayed spectrophotometrically using ferricytochrome *c* as the terminal electron acceptor (oxidant) by a modified method of Obidoa *et al.* (1978). The reaction medium (final volume of 3.0 ml) consisted of 100 mM potassium phosphate buffer pH 7.4, 1.33 μM ferricytochrome *c*; and 2.5 mg mitochondrial protein preincubated for 2 min in freshly neutralized 20 mM potassium cyanide to inhibit the cytochrome oxidase. The concentrations indicated were the final concentrations in the reaction medium. The effects of the toxins on the enzyme activities were determined by adding the appropriate amounts of the toxins to the reaction medium, while 10% N,N^1-dimethyl formamide (DMF) solution of equivalent volume was added in control experiments. The reaction was initiated by adding either succinate (final concentration in reaction medium was 20 mM) for succinate-cytochrome *c* reductase, or *a*-ketoglutarate (final concentration in reaction medium was also 20 mM) for *a*-ketoglutarate cytochrome *c* reductase, to the reaction medium. Oxidized NAD^+ (final concentration in reaction medium was 10 mM) was added as a coenzyme prior to the addition of *a*-ketoglutarate in the *a*-ketoglutarate cytochrome *c* reductase assay. Absorbance changes of the assay medium were recorded at 550 nm for 6 min in a Milton Roy Spectronic 1201 UV-visible split beam spectrophotometer set at the kinetic mode. Specific activities of the enzyme (v) were calculated from the following relationship (Obi, 1985).

$v = \frac{OD/t}{PE}$, where

P = protein concentration,

E = ferricytochrome *c* extinction coefficient (18.5 M^{-1} cm^{-1})

OD/t = absorbance change per unit time.

Enzyme activity (v) was expressed as nmol Fe^{3+} cytochrome *c* reduced per minute per mg mitochondrial protein (mmp). i.e., nmol Fe^{3+} Cyt *c* reduced $\times$ min^{-1} $\times$ mmp^{-1}.

Preincubation experiments were carried out by first incubating the appropriate amount of the coumarin (scopoletin or AFB_1) compound to be tested (or equivalent volume of aqueous 10% DMF solution for control), with the needed amount of mitochondrial suspension for 2–3 min before the other components of the reaction medium were added.

All other assay procedures were as described earlier.

Analysis

All statistical analysis were done by Students "t" test.

Results and Discussion

Effects of Scopoletin and AFB_1 on Bovine Hepatic Mitochondrial a-ketoglutarate cytochrome c reductase

Both scopoletin and AFB_1 stimulated bovine hepatic mitochondrial *a*-ketoglutarate cytochrome *c* reductase at the toxin concentrations (5–20 nmol/mmp) studied.

However, the stimulatory effects of scopoletin (8–33%) were higher than those of AFB_1 (5–23%) at equivalent concentration (Table I). Apart from the effects of the 10 nmol/mmp concentrations of the toxins, the difference between the effects of scopoletin and AFB_1 on *a*-ketoglutarate-cytochrome *c* reductase were not significant ($p>0.05$). However, the stimulatory effects of both scopoletin and AFB_1 on the enzyme were significant ($p<0.05$) relative to the control from 10–20 nmol/mmp concentrations.

Table I. % Change in the mean activities of bovine hepatic mitochonrial *a*-ketoglutarate-cytochrome *c* reductase in the presence of scopoletin and aflatoxin B_1 (AFB_1).

	Non-preincubated	Preincubated
* Mean activity (Δv) in the absence of the coumarins (10% DMF) control experiment	5.08 ± 1.21	5.12 ± 0.67
% change in activity due to 5 nmol scopoletin per mmp	+ 8.0	+13.9
% change in activity due to 10 nmol scopoletin per mmp	+20.1	+33.8
% change in activity due to 20 nmol scopoletin per mmp	+33.2	+50.6
% change in activity due to 5 nmol AFB1/mmp	+ 5.0	+28.0
% change in activity due to 10 nmol AFB1/mmp	+10.4	+49.0
% change in activity due to 20 nmol AFB1/mmp	+22.9	+58.8

The mean activities(velocity) are expressed as nmole $Fe^{3+}Cyt$ reduced $\times$ min^{-1} $\times$ mmp^{-1}.
The values were calculated in the presence of different concentrations (5–20 nmol/mmp of scopoletin and AFB_1, at fixed substrate concentration [S] of 1.33 μM. Enzyme activities are expressed as mean ± S.D(n=6). % change in activities due to the coumarins were calculated from the expressions $\frac{\mathbf{B - A}}{A} \times 100$ where A and B are mean enzyme activities in the absence and presence of the coumarins, respectively. + sign indicates stimulation.

Table II. Summary of the effects of scopoletin and aflatoxin B_1 on the kinetic parameters (K_m and V_{max}) of bovine hepatic mitochondrial *a*-ketoglutarate cytochrome *c* reductase.

Concentration of the compounds [nmol/mmp]	K_m [μM]	V_{max} (nmol Fe^{3+}Cyt.*c* red $\times$ min^{-1} $\times$ mmp^{-1}
Control (10%DMF)	2.10±0.95	5.55±1.02
5 nmol scopoletin	0.60±0.05	5.50±1.21
10 nmol scopoletin	0.90±0.14	6.15±0.93
20 nmol scopoletin	0.59±0.02	6.00±1.10
5 nmol AFB_1	1.40±0.86	5.10±1.14
10 nmol AFB_1	1.25±0.42	5.80±1.28
20 nmol AFB_1	1.10±0.16	6.00±0.25

The values represent mean ±S. D. (n=6).

Changes of the kinetic parameters (K_m and V_{max}) calculated by direct liner plots according to Eisenthal and Cornish-Bowden (1974) are shown in Table II. The results show that in the presence of the toxins, the K_m values generally decrease relative to the control ($p<0.05$), while the V_{max} slightly increased ($p>0.05$).

These effects were concentration-dependent, and more pronounced with scopoletin than with AFB_1. This is in agreement with the observed stimulatory effects of the toxins.

However, the values for the lower AFB_1 concentrations (5–10 nmol/mmp) indicated decreases relative to the control values.

On pre-incubating the toxins with mitochondria and assaying for the enzyme activity, an enhancement of the stimulatory effects of both AFB_1 and scopoletin relative to the non-preincubated condition was observed. The increases in the enzyme activities due to pre-incubation were generally significant ($p<0.05$) relative to the corresponding non pre-incubated values. The enhancement was more for AFB_1 (20–39% increase) than for scopoletin (5–23% increase).

Effects of scopoletin and AFB1 on bovine hepatic mitochondrial succinate cytochrome c reductase

The bovine hepatic mitochondrial succinate-cytochrome *c* reductase was generally inhibited by both scopoletin and AFB_1. However, the inhibitory effects of scopoletin on the enzyme were found to be inversely proportional to the toxin concentration. On the other hand, the inhibitory effects of AFB_1 on the enzyme was directly proportional to the toxin concentration although the lower (5.0 nmol/mmp) concentration of the toxin exhibited small stimulatory effects on the enzyme (Table III).

At equivalent concentrations (10–20 nmol/mmp), AFB_1 exhibited higher inhibitory effects (10–20%) on the succinate-cytochrome *c* reductase than scopoletin (1–8%).

Table III. % Change in the mean activities of bovine hepatic mitochondrial succinate-cytochrome *c* reductase in the presence of scopoletin and aflatoxin B_1.

* Mean activity ($\Delta\nu$) in the absence of the coumarins (10% DMF)	Non-preincubated	Preincubated
Control experiment	5.9 + 1.35	87 + 1.20
% change in activity due to 5 nmol scopoletin/mmp	−8.5	+5.1
% change in activity due to 10 nmol scopoletin/mmp	−7.2	+2.4
% change n activity due to 20 nmol scopoletin/mmp	−1.0	+6.8
% change in activities due to 5 nmol AFB1/mmp	+2.0	0
% change in activities due to 10 nmol AFB1/mmp	−10.2	+15.0
% change in activities due to 20 nmol AFB1/mmp	−18.2	+16.0

The mean activities (velocity) are expressed as nmol Fe^{3+} Cyt.*c* red × min^{-1} x mmp^{-1}. The values were calculated in the presence of different concentrations (5–20 nmol/mmp) of scopoletin and AFB_1, at a fixed substrate concentration [S] of 1.33 μM. Enzyme activities are expressed as mean ± S. D. (N=6). % change in activity were calculated as indicated in Table I legend.
+ Sign indicates stimulation; while − sign indicates inhibition.

Although these inhibitions were generally not significant (P>0.05) except apparently, for that of 20 nmol/mmp AFB_1, the inhibitory effects of AFB_1 on the enzyme were generally significantly (P<0.05) higher than those of scopoletin at equivalent concentrations of the toxins.

Changes in the kinetic parameters (K_m and V_{max}) as shown in Table IV, indicate a general increase in the K_m values of the enzyme in the presence of the toxins. The calculated V_{max} from these plots, however, indicated values consistent with the observed inhibitory/stimulatory (for 5 nmol/mmp AFB_1) effects of AFB_1 on the enzyme. On the other hand, the calculated V_{max} values for higher scopoletin concentrations (10–20 nmol/mmp) deviated from the observed inhibitory effects. Likewise, the calculated K_m values in the presence of 5 nmol/mmp AFB_1 was not consistent with the observed stimulatory effects (higher V_{max} values).

Pre-incubating the mitochondria with the toxins generally reversed the inhibitory/stimulatory effects of the various concentrations of both toxins (Table III).

Table IV: Summary of the effects of scopoletin and aflatoxin B_1 on the kinetic parameters (K_m and V_{max}) of bovine hepatic mitochondrial succinate cytochrome *c* reductase.

Concentrations of the compounds [nmol/mmp]	K_m [μM]	V_{max} (nmol Fe^{3+} Cyt *c* red × min^{-1} x mmp^{-1})
Control (10% DMF)	1.40±0.82	6.25±1.20
5 nmol Scopoletin	1.80±0.56	5.80±1.61
10 nmol Scopoletin	2.40±0.25	6.40±1.72
20 nmol Scopoletin	1.60±0.34	6.50±0.95
5 nmol AFB_1	2.20±0.45	7.0±1.95
10 nmol AFB_1	1.50±0.32	5.65±0.98
20 nmol AFB_1	3.1±0.76	5.90±0.75

The values represent mean ±S. D. (*n*=6).

These results showed that by using kinetic studies, it could be established that both scopoletin and AFB_1 stimulated bovine hepatic mitochondrial NAD^+-linked *a*-ketoglutarate cytochrome *c* reductase but inhibited the FAD^+-linked succinate cytochrome *c* reductase activities *in vitro*. The increase in the stimulatory effects of the toxins on *a*-ketoglutarate cytochrome *c* reductase, and the reversal of their inhibitory effects on succinate cytochrome *c* reductase by prolonged exposure of the mitochondria to the toxins (pre-incubation) emphasizes the important roles which the membrane plays on the effects of these toxins, since succinate dehydrogenase is membrane bound and *a*-ketoglutarate dehydrogenase is located in the matrix. These effects could result from the symmetric orientation, restriction, and plasticity of membrane bound enzymes as indicated by Obidoa *et al.* (1980). Consequently, the penetration of the mitochondrial membrane by these toxins as had been suggested (Obasi and Obidoa, 1997b) could cause alterations in the molecular architecture or orientation and function of the membrane bound proteins such as succinate dehydrogenase enzyme proteins. These observations could however, be confirmed by the assay of the purified enzymes alone and in reconstituted systems.

When taken with our previous reports on the effects of these toxins on the bovine hepatic mitochondrial respiration, and complex I enzymes (Obasi and Obidoa, 1995, 1997a), these results further indicate that at very minute levels, scopoletin and AFB_1 could elicit alterations in the bovine hepatic mitochondrial respiratory activities.

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