Vol. 82, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1978 Pages 348-355

7,8-BENZOFLAVONE STIMULATES THE METABOLIC ACTIVATION OF AFLATOXIN B_1 TO MUTAGENS BY HUMAN LIVER

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Received March 9,1978

SUMMARY: The addition of 7,8-benzoflavone to a monooxygenase system from human liver markedly stimulated the metabolic activation of aflatoxin B_1 to mutagens. When 7,8-benzoflavone (5×10^{-5}M) was added to this monooxygenase system, the amount of aflatoxin B_1 needed for a mutagenic response was decreased by 20- to 40-fold. 7,8-Benzoflavone did not stimulate the metabolic activation of aflatoxin B_1 to mutagens when rat liver was used as a source of monooxygenase.

Earlier studies from our laboratory indicated that addition of 7,8-benzoflavone to homogenates of human liver increased the rates of hydroxylation of benzo(a)pyrene, zoxazolamine, and antipyrine but had little or no effect on the oxidative metabolism of 7-ethoxycoumarin, coumarin, or hexobarbital (1). The present study was initiated to determine whether the in vitro addition of 7,8-benzoflavone to a liver monooxygenase system could stimulate the metabolic activation of aflatoxin B_1 to reactive intermediates that cause mutations in strains TA 98 and TA 100 of Salmonella typhimurium. The results presented here indicate that 7,8-benzoflavone markedly stimulates the metabolic activation of aflatoxin B_1 by human liver.

MATERIALS AND METHODS: Human liver samples were obtained by surgical biopsy from patients undergoing abdominal surgery for cancer. One portion of the biopsy sample was subjected to histological examination. The remainder of the sample was stored in liquid nitrogen. Only samples with normal liver histology were used for our studies. Liver was also obtained from untreated, immature male rats of the Long-Evans strain. Sterile technique and materials were used to prepare homogenate, supernatant fraction, and microsomes. Homogenates (20% w/v in 0.25 M sucrose and 0.05 M Tris HCl buffer, pH 7.4) were centrifuged at 12,000 x g for 20 min. In some experiments the supernatant fraction was used to prepare microsomes, and in other experiments the 12,000 x g supernatant fraction was divided into aliquots and stored in liquid nitrogen.

0006-291X/78/0821-0348$01.00/0

Strains TA 98 and TA 100 of S. typhimurium (2) were obtained from Dr. B. Ames (University of California, Berkeley, CA). Complete procedures for inducing mutations in these bacteria have been described (3,4). In our study, the total incubation volume of 0.50 ml for each reaction contained 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP, 100 mM sodium phosphate (pH 7.4), 10^8 bacteria, liver supernatant fraction, the flavone compound, and aflatoxin B_1. When microsomes were used instead of the supernatant fraction, 1 unit glucose-6-phosphate dehydrogenase was added to the incubation mixture. Flavone compounds were added in 10 µl dimethylsulfoxide, and aflatoxin B_1 (Calbiochem, San Diego, CA) was added in 20 µl dimethylsulfoxide. Appropriate amounts of vehicle were added to control samples. After the monooxygenase system and aflatoxin B_1 had incubated 5 min at 37°, 2 ml of top agar was added, and the mixture was poured onto petri plates. Mutation frequency was assessed 48 hr later by counting the macroscopic colonies of bacteria on the plates. Each value represents the average from triplicate plates. 7,8-Benzoflavone and 5,6-benzoflavone were purchased from Aldrich Chemical Co. (Milwaukee, WI), and 4'-bromoflavone was a gift from Dr. L. Wattenberg (University of Minnesota, Minneapolis, MN).

RESULTS: In strain TA 98 of S. typhimurium, the number of histidine revertants resulting from the metabolic activation of aflatoxin B_1 was related to the concentration of human liver in the incubation mixture (Fig. 1). In complete incubations containing cofactors, aflatoxin B_1, and 12,000 x g supernatant fraction or microsomes from human liver, 7,8-benzoflavone (5×10^{-5}M) markedly increased the number of histidine revertants (Figs. 1 and 2). The maximum stimulation of the metabolic activation of aflatoxin B_1 to mutagens by human liver was observed with 10^{-5} to 10^{-4}M 7,8-benzoflavone (Fig. 2). Although 10^{-6}M 7,8-benzoflavone did not influence the metabolic activation of aflatoxin B_1 in the experiment described in Fig. 2, the number of histidine revertants was decreased when some human liver samples were incubated with low concentrations of aflatoxin B_1 and 10^{-6}M 7,8-benzoflavone.

7,8-Benzoflavone had no intrinsic mutagenicity (Fig. 1) and was not metabolized to mutagens (Fig. 3). In incubations without aflatoxin B_1 or human liver, the spontaneous mutation frequency with S. typhimurium strain TA 98 was 20-30 histidine revertants. When 7,8-benzoflavone (5×10^{-5}M) was added to incubations without aflatoxin B_1 and without human liver, the mutation frequency remained at the spontaneous level. When 7,8-benzoflavone was added to incubations without aflatoxin B_1 but with enzymes from human liver and cofactors for metabolism, the mutation frequency also remained at the spontaneous level.

Several flavone compounds stimulated the metabolic activation of aflatoxin B_1 to mutagens by human liver. Like 7,8-benzoflavone, 5,6-benzoflavone and 4'-bromofla-

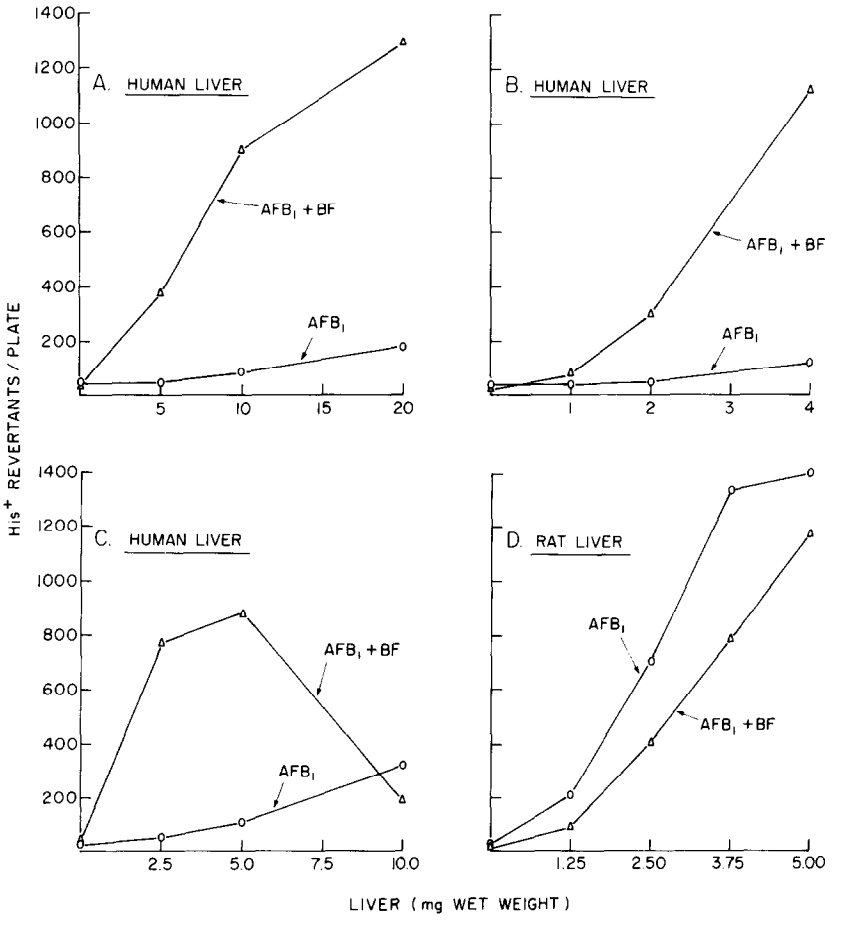

Fig. 1. Effect of 7,8-benzoflavone on the metabolic activation of aflatoxin B_1 to mutagens by several concentrations of 12,000 x g supernatant fraction from human or rat liver. Incubation mixtures contained an NADPH-generating system, S. typhimurium strain TA 98, varying amounts of 12,000 x g supernatant fraction from human or rat liver, and the following amounts of aflatoxin B_1 (AFB_1): 1.5 nmol, A; 2.5 nmol, B; and 5 nmol C and D. 7,8-Benzoflavone (BF) was added to give a final concentration of 5×10^{-5} M in the incubation mixture. In control incubations without aflatoxin B_1, the addition of 7,8-benzoflavone did not increase the histidine revertants above the spontaneous level of 20-30 revertants per plate either in the absence or presence of liver enzymes.

vone were not intrinsically mutagenic nor were they metabolized to mutagenic products; however, these flavones all stimulated the metabolic activation of aflatoxin B_1 severalfold. The greatest effect was observed with 7,8-benzoflavone, which was 2- to 5-fold more active than the other two flavones.

Stimulation of the metabolic activation of aflatoxin B_1 by 7,8-benzoflavone can result in toxicity and bacterial death. Fig. 1C shows that 7,8-benzoflavone markedly

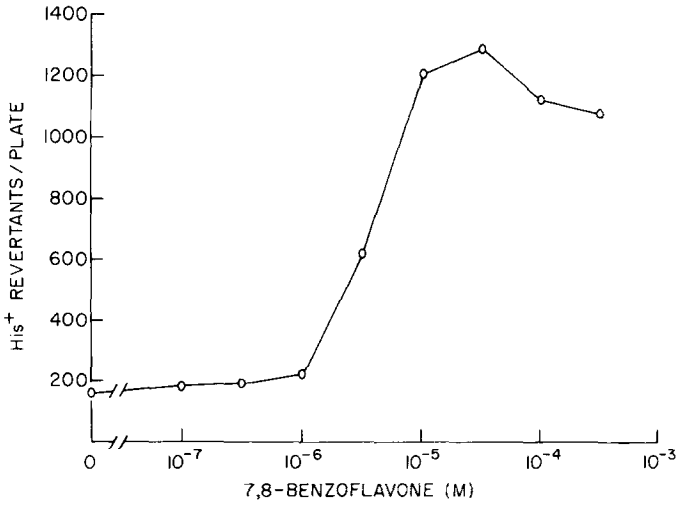

Fig. 2. Effects of varying concentrations of 7,8-benzoflavone on the metabolic activation of aflatoxin B_1 to mutagens by microsomes from human liver. Incubation mixtures contained an NADPH-generating system, S. typhimurium strain TA 98, aflatoxin B_1 (10 nmol), and microsomes (0.2 mg protein) from human liver.

increased the number of histidine revertants with 2.5 and 5 mg of wet weight liver. With 10 mg of liver, however, toxicity resulted and few revertant colonies were observed. Therefore, studies on the effects of flavones on the metabolic activation of aflatoxin B_1 must be done with low concentrations of tissue and/or low concentrations of aflatoxin B_1.

To obtain a quantitative estimate of how much stimulation of the metabolic activation of aflatoxin B_1 occurs when 7,8-benzoflavone is added to the incubation mixture, an experimental design commonly used to compare the potency of different compounds in the Salmonella test-system was adopted. By adding a constant amount of human liver and varying the amount of aflatoxin B_1 from 0.1 to 10 nmol, a relatively linear dose vs. mutagenic response relationship was observed in the absence of 7,8-benzoflavone (Fig. 3). In the presence of 7,8-benzoflavone, a linear dose response was observed only with low concentrations of aflatoxin B_1, and toxicity was observed at higher concentrations. Fig. 3A shows that in S. typhimurium strain TA 98, 700 revertants were observed when 0.25 nmol of aflatoxin B_1 was incubated with human liver in the presence of 5×10^{-5}M 7,8-benzoflavone, whereas incubation of 9 nmol of aflatoxin B_1 was required to obtain 700 revertants when the metabolism of aflatoxin B_1 occurred in the absence of

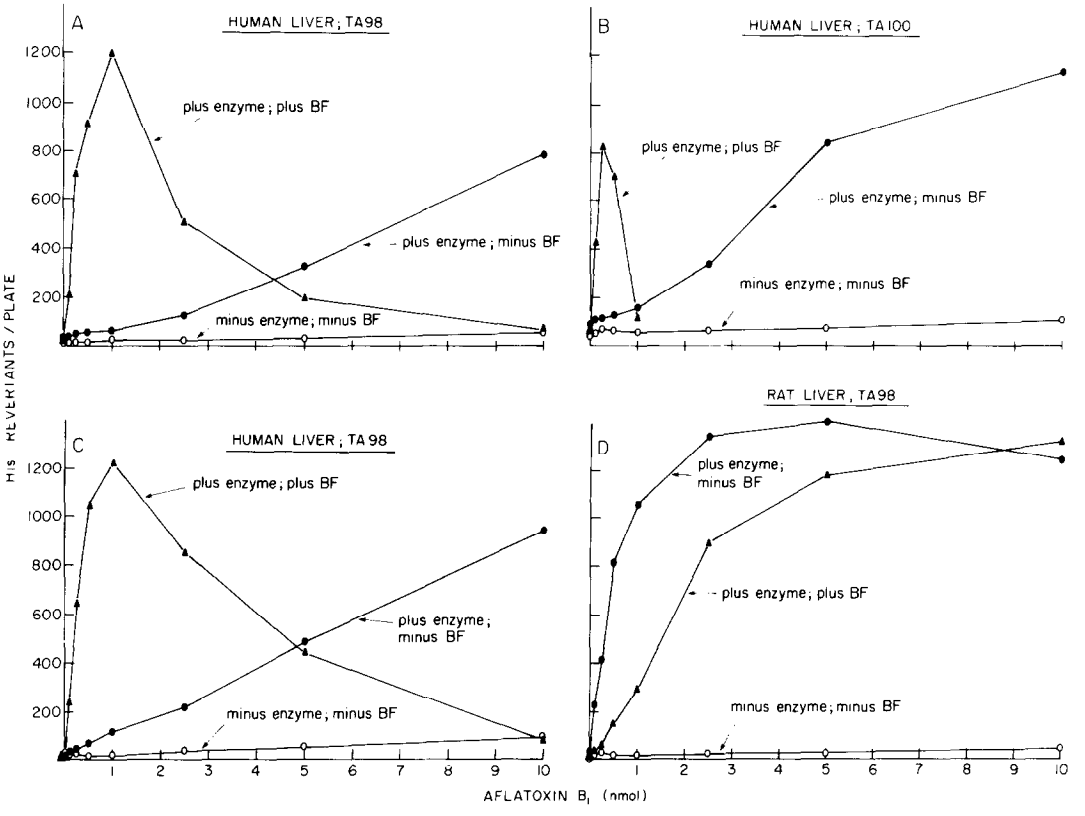

Fig. 3. Effect of 7,8-benzoflavone on the metabolic activation of varying concentrations of aflatoxin B_1 by 12,000 x g supernatant fractions from human or rat liver. Incubation mixtures contained an NADPH-generating system, S. typhimurium strain TA 98 or TA 100, aflatoxin B_1, and the 12,000 x g supernatant fraction from 10 mg wet weight human liver sample 1, A and B; from 5 mg human liver sample 2, C; and from 5 mg rat liver, D. 7,8-Benzoflavone (BF) was added as indicated to give a final concentration of 5×10^{-5}M in the incubation mixture. As shown by the low number of histidine revertants in the absence of aflatoxin B_1, 7,8-benzoflavone was not mutagenic nor was it metabolized to mutagens.

7,8-benzoflavone. In the same experiment using S. typhimurium strain TA 100 and the same human liver sample (Fig. 3B), 420 revertants were observed when 0.125 nmol of aflatoxin B_1 was incubated in the presence of 5×10^{-5}M 7,8-benzoflavone, whereas incubation of 3 nmol of aflatoxin B_1 was required to obtain 420 revertants when the metabolism of aflatoxin B_1 occurred in the absence of 7,8-benzoflavone. The results in Fig. 3, and similar results with several other human liver samples, indicate that the metabolic activation of aflatoxin B_1 to mutagens by human liver was enhanced 20- to 40-fold by 5×10^{-5}M 7,8-benzoflavone. In contrast to these results, when rat liver was used

as a source of monooxygenase, the metabolic activation of aflatoxin B_1 to mutagens was inhibited by 2- to 5-fold (Figs. 1D and 3D).

DISCUSSION: The pioneering research of Drs. James and Elizabeth Miller and their associates has indicated that aflatoxin B_1 is metabolized to aflatoxin B_1-2,3-oxide, which is covalently bound to nucleic acid (5-8), and recent studies by Essigmann et al. have also indicated that aflatoxin B_1 is metabolized to aflatoxin B_1-2,3-oxide which binds to DNA (9). This oxide of aflatoxin B_1 is believed to cause cancer, mutations and others cellular toxicities. The stimulatory effect of 7,8-benzoflavone on the metabolic activation of aflatoxin B_1 to mutagens by human liver (Figs. 1-3) suggests that 7,8-benzoflavone may enhance the metabolism of aflatoxin B_1 to aflatoxin B_1-2,3-oxide in human liver.

With human liver samples, 7,8-benzoflavone stimulates the metabolic activation of aflatoxin B_1 to mutagens (described above) and the rates of hydroxylation of benzo(a)pyrene, zoxazolamine, and antipyrine (1). These results suggest that the metabolism of all four substrates is catalyzed by the same or similar monooxygenase systems. Because 7,8-benzoflavone has little or no effect on the rates of the oxidative metabolism of 7-ethoxycoumarin, coumarin, and hexobarbital in human liver samples (1), the enzyme systems that metabolize these substrates seem to be different from the monooxygenases that metabolize aflatoxin B_1. Although the addition of 7,8-benzoflavone to human liver stimulates the metabolic activation of aflatoxin B_1 and the hydroxylation of benzo(a)pyrene, zoxazolamine, and antipyrine, little or no stimulation in the metabolism of these substances occurred when 7,8-benzoflavone was added to rat liver (Figs. 1 and 3, and unpublished observations). These results suggest that monooxygenases in rat liver are different from those in human liver.

The in vitro effect of 7,8-benzoflavone and other flavones as modulators of benzo(a)pyrene metabolism has been studied by several investigators (10-16). Other chemicals that activate monooxygenase reactions include ethyl isocyanide and acetone which activate aniline hydroxylation (17,18), metyrapone which activates acetanilide hydroxylation (19), and certain carcinogens which activate the 2-hydroxylation of biphenyl (20).

The stimulation of the hydroxylation of several drugs, as well as the metabolic activation of aflatoxin B_1 by 7,8-benzoflavone, suggests that this compound may enhance the ability of human liver to metabolically activate other chemicals to mutagens. These data also suggest that 7,8-benzoflavone may increase our capacity to detect premutagens that require metabolic activation to reactive intermediates. Recent studies have indicated that the indole derivative norharman enhances the mutagenicity of aniline and other premutagens when a rat liver monooxygenase and S. typhimurium strain TA 98 was used; however, norharman was inactive when S. typhimurium strain TA 100 was used to detect mutations (21). The results of our studies indicate that 7,8-benzoflavone enhances the metabolic activation of aflatoxin B_1 by human liver in the presence of either S. typhimurium strain TA 98 or TA 100, but the metabolic activation of aflatoxin B_1 to mutagens did not occur when rat liver was used as a source of monooxygenase. Because 7,8-benzoflavone and other synthetic flavones enhance the metabolic activation of aflatoxin B_1 by human liver, additional studies are needed to determine whether some of the naturally occurring flavones can enhance the metabolic activation of aflatoxin B_1 and other premutagens.

ACKNOWLEDGMENT: We thank Mary Kay Barrett of the Memorial Sloan-Kettering Cancer Center for her help with this study. We thank Arlene Ott and MaryAnn Augustin for their help in preparing this manuscript.

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