Effect of Endurance Exercise Training on Aflatoxin B₁ Hepatotoxicity in Rats

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Summary Effect of endurance exercise training on hepatotoxicity induced by aflatoxin B₁ (AFB₁) was studied in rats. Rats were subjected to swimming with 1%BW resistance for 30 min, 5 days/week for 14 weeks before administration of AFB₁. Endurance exercise training induced high physical fitness as shown by reduction in resting heart rate and increase in the activities of mitochondrial succinate dehydrogenase and citrate synthase in the gastrocnemius muscle. Water-immersed rats had similar basal physical fitness when compared with that of the untrained rats. Endurance exercise training as per the above schedule followed by a single i.p. injection of AFB₁ (2 mg/kg BW) caused a significant increase in the activities of serum alanine aminotransferase (ALT) by 6.6 fold and aspartate aminotransferase (AST) by 1.8 fold and increased the severity of histopathologic hepatic necrosis at 24 h after AFB₁ administration. Endurance exercise training potentiated AFB₁-induced hepatotoxicity by increasing the activity of the hepatic monooxygenase enzymes aniline hydroxylase and *p*-nitroanisole-O-demethylase. These results suggest that potentiation of AFB₁-hepatotoxicity by endurance exercise training may be due to an increase in the metabolic formation of AFB₁-8,9-oxide, which, in turn, causes a marked increase in AFB₁ binding to hepatic DNA and proteins.

Key Words: endurance exercise, aflatoxin B₁, liver injury, drug-metabolizing enzymes, lipid peroxidation

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Chronic physical training produces various types of physiological adaptation that depend on the type of exercise stimulus. There is evidence to support the view that endurance training has a positive impact on many organ functions in promoting good health, especially on cardiovascular and musculoskeletal systems [1]. Endurance training is considered to be a strategy for disease prevention, especially against myocardial infarction, musculoskeletal injury, etc., because it contributes to increased strength and muscle elasticity, decreased cholesterol in blood, and increased myocardial capillarization [1, 2]. These changes work in concert for improvement of health.

Aflatoxins are naturally occurring toxic metabolites produced by *Aspergillus flavus* and other related fungal species. Aflatoxin B₁ (AFB₁) is the most studied of the naturally occurring aflatoxins and the most biologically active. It has been found to be a potent hepatotoxic, hepatocarcinogenic, teratogenic, and mutagenic mycotoxin in a variety of *in vivo* and *in vitro* systems [3]. It is of considerable concern that many food items consumed around the world, such as peanuts, rice, grains, corn, beans, and sweet potatoes, are often contaminated with AFB₁.

It is likely that the hepatotoxicity of AFB₁ contained in foods might be modified in persons who have been exercise training. Therefore, it is of interest to investigate the effect of endurance exercise training on the hepatotoxicity of AFB₁ in rats and the possible mechanisms of this effect.

MATERIALS AND METHODS

Materials. Aflatoxin B₁ and all other reagents were purchased from Sigma Chemical Co., St. Louis, MO.

Animals. Male Wistar rats (200–240 g) supplied from the National Animal Center, Mahidol University, were housed in stainless steel cages in a room approximately $25 \pm 2^\circ\text{C}$ with relative humidity at about 65%. The animals were fed regular rat chow (Gold Coins Co., Singapore) and given water *ad libitum*.

Endurance exercise training. The exercise program was designed to improve the fitness and to be sufficient to produce training effects in the rats. Rats were subjected to a swimming program of 30 min, once a day, 5 days/week, for 14 weeks. They swam with a weight resistance of 1% of their body weight fixed to their tails. The animals swam in a cylindrical plastic tank that had a diameter and height about 41 cm by 60 cm, respectively, and contained 32 liters of water at a temperature of about 26°C . The water level was high enough so that the rats' tails did not reach the bottom of the tank. The tap water was allowed to stand in the tank for about 1 day before use to reduce the buoyant effect of trapped air in the fur of the rat.

Effect of endurance exercise training on physiological adaptation. Rats were divided randomly into 3 groups: untrained, untrained-water immersion, and trained groups. Rats in the untrained group were control animals, and they were only housed in a cage. In the untrained-water immersion group, rats were put in

a cage and immersed in water reaching their upper chests. Rats in the trained group were trained with the exercise program as described above. Resting heart rate was measured in all groups at 24 h after the last training session. The animals were anesthetized with ether, and blood was collected at 48 h after the last training. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured with a Sigma Kit (Sigma Chemical Co.). Albumin and globulin concentrations were determined with a BM Lab Kit (BM Laboratory, Thailand).

Gastrocnemius muscles were quickly excised, weighed, and immersed in ice-cold 0.25 M sucrose. The muscular homogenate was then prepared, and the activities of mitochondrial succinate dehydrogenase [4] and citrate synthase [5] were determined. The heart was excised and opened to remove blood clots in the atria and ventricles; then the fat and connective tissue were trimmed off and the organ was weighed. The liver was perfused with ice-cold 0.9% NaCl, weighed, and then homogenized. Hepatic lipid peroxide [6], triglyceride [7], and glutathione [8] were determined. The activities of aniline hydroxylase [9] and *p*-nitroanisole-O-demethylase [10] in the microsomal fraction, and the activity of glutathione-S-transferase [11] in the cytosolic fraction, of the liver were also determined. Protein content was measured by the method of Lowry *et al.* [12].

Effect of endurance exercise training on hepatotoxicity of AFB₁. Rats were randomly divided into 4 groups. Groups I and II were left untrained and given the training schedule, respectively, as previously described, prior to a single i.p. administration of DMSO. Groups III and IV were untrained and trained, respectively, prior to a single i.p. administration of AFB₁ (2 mg/kg BW). AFB₁ was administered to the rats at 48 h after the last training. At 24 h after AFB₁ administration, the rats were sacrificed, and blood was collected for the determination of serum albumin and globulin and the activities of serum ALT and AST. Livers were collected for the determination of hepatic lipid peroxide, triglyceride, and glutathione contents. A portion of each liver was fixed for histopathologic examination.

Statistical analysis. Statistical evaluation was carried out by analysis of variance (ANOVA), and the differences between pairs of means were evaluated by the Least Significance Difference (LSD) test. Differences between groups were considered significant at the $p < 0.05$ two-tailed level.

RESULTS

Effect of endurance exercise training on the indices of endurance training

The indices of endurance exercise training in rats after 14 weeks of swimming training are summarized in Table 1. We found that the increment of body weight in the trained group was significantly lower than that in the immersed group by 29.9% ($p < 0.001$), and lower than that in the untrained group by 28.3% ($p < 0.001$). The relative heart weight was 30% greater in the trained group than in the

Table 1. Indices of endurance exercise training in rats measured after exercise training for 14 weeks.

Parameter	Condition		
	Untrained group	Untrained-immersed group	Trained group
Body weight increment (g)	255 ± 7	261 ± 9	183 ± 5***,b
Heart weight (mg/g BW)	2.23 ± 0.09	2.24 ± 0.08	2.91 ± 0.01***,b
Resting heart rate (beats/min)	350 ± 14	325 ± 13	303 ± 4 ^a
Succinate dehydrogenase (ng formazan/mg tissue/min)	8.22 ± 0.87	8.85 ± 1.30	12.3 ± 0.79*, ^a
Citrate synthase (nmol mercaptide/g tissue/min)	73.8 ± 7.5	131 ± 11	175 ± 13***,b

Values are means ± SEM of 8–10 animals. Body weight increment was evaluated from the difference of body weight between 1st week and 15th week. Succinate dehydrogenase and citrate synthase activities in the mitochondria of gastrocnemius muscle were measured. Significant values are indicated by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant difference between trained and untrained-immersed groups; ^a $p < 0.01$, ^b $p < 0.001$, significant difference between trained and untrained groups.

immersed and untrained groups ($p < 0.001$). The resting heart rate in trained group was lower than that in the untrained group by 13.5% ($p < 0.001$).

The activity of succinate dehydrogenase was greater in the trained group than in the immersed and untrained groups by 38.9% ($p < 0.05$) and 49.6% ($p < 0.01$), respectively. That of citrate synthase in the trained group was also greater, by 33.6% ($p < 0.01$) and 137% ($p < 0.001$), than the activity in immersed and untrained groups, respectively.

There were no significant differences in any parameters between untrained and untrained-immersed groups. However, water immersion tended to cause an increase in the activity of citrate synthase.

Effect of endurance exercise training on liver and serum composition

The liver and serum composition in rats after 14 weeks of swimming training are summarized in Table 2. Endurance exercise training did not cause any changes in liver weight or hepatic triglyceride, glutathione, and lipid peroxide content. In addition, serum albumin and globulin and the activity of serum AST were not altered by the training. However, the activity of serum ALT in the trained group was slightly, but significantly, increased, by 32.7% ($p < 0.05$) and 29.1% ($p < 0.01$), when compared with that activity in the immersed and untrained groups, respectively. Water immersion did not cause any significant changes in the above parameters.

The hepatic drug-metabolizing enzyme systems in rats after 14 weeks of

Table 2. Effect of endurance exercise training for 14 weeks on the liver and serum composition in rats.

Parameter	Condition		
	Untrained group	Untrained-immersed group	Trained group
Liver			
Liver weight (mg/g BW)	20.4±0.8	21.9±1.5	22.3±0.4
Triglyceride (mg/g liver)	7.45±0.77	8.83±0.87	6.78±0.76
Lipid peroxide content (nmol MDA/100 mg protein)	189±27	175±16	198±22
Microsomal protein (mg/g liver)	18.4±1.2	16.7±1.0	16.2±1.4
Cytosolic protein (mg/g liver)	60.8±2.4	67.6±1.6	64.5±1.7
Glutathione-S-transferase (nmol DCNB/mg protein/min)	1.18±0.06	1.18±0.05	1.33±0.06
Glutathione (μmol/g liver)	4.47±0.20	4.75±0.19	4.46±0.10
Serum			
Albumin (g%)	4.34±0.20	4.03±0.23	4.08±0.11
Globulin (g%)	2.68±0.12	2.67±0.78	2.71±0.08
AST (IU/ml)	173±24	197±35	182±14
ALT (IU/ml)	22.3±1.2	21.7±2.9	28.8±2.1 ^{*,a}

Values are means±SEM of 8–10 animals. Significant values are indicated by asterisks: * $p < 0.05$, significant difference between trained and untrained-immersed groups; ^a $p < 0.05$, significant difference between trained and untrained groups.

Fig. 1. Effect of endurance exercise training for 14 weeks on the activities of hepatic microsomal aniline hydroxylase and *p*-nitroanisole-O-demethylase in rats. Values are means±SEM of 8–10 animals. Significant values are indicated by asterisks: *** $p < 0.001$, significant difference between trained and untrained-immersed groups; ^a $p < 0.001$, significant difference between trained and untrained groups.

swimming are also summarized in Table 2 and Fig. 1. Microsomal and cytosolic proteins were not altered by endurance exercise training. Cytosolic glutathione-S-transferase activity was slightly increased by, 12.7%, in the trained group when compared with the value for the untrained-immersed group. However, endurance exercise training caused a substantial increase in the activity of aniline hydroxylase, by 87.3% ($p < 0.001$) and 111% ($p < 0.001$), when the training group was compared with immersed and untrained groups. The activity of *p*-nitroanisole-O-demethylase in the trained group was also significantly higher, by 75.5% ($p < 0.001$) and 95.4% ($p < 0.001$), than that of the immersed and untrained groups. Water immersion itself did not affect any of these parameters.

Effect of endurance exercise training on the hepatotoxicity of AFB₁

Hepatic lipid peroxide and serum AST and ALT activities in rats given swimming training for 14 weeks and then treated with a single i.p. dose of AFB₁ are shown in Fig. 2. At 24 h after AFB₁ treatment, the lipid peroxide level in the liver of untrained rats treated with AFB₁ was significantly increased by 4.2 fold ($p < 0.001$) in comparison with that for untrained rats treated with DMSO; whereas in trained rats, the lipid peroxide level in those treated with AFB₁ was only slightly greater than in those treated with DMSO. Thus the lipid peroxide level in trained rats treated with AFB₁ was significantly lower, by 3.8 fold ($p < 0.001$), than that in untrained rats treated with AFB₁. Serum AST and ALT activities were significantly increased in both trained and untrained rats treated with AFB₁ in comparison with those activities of rats treated with DMSO. At 24 h after AFB₁ treatment, serum AST and ALT activities were significantly increased by 1.8 fold ($p < 0.05$) and 6.6 fold ($p < 0.001$), respectively in trained rats when compared with

Fig. 2. Effect of endurance exercise training for 14 weeks on the hepatic lipid peroxide level and the activities of serum AST and ALT measured 24 h after treatment with AFB₁ (2 mg/kg BW) in rats. Values are means $\pm$ SEM of 8–10 animals. Significant values are indicated by asterisks: * $p < 0.05$, *** $p < 0.001$, significant difference between untrained-AFB₁ and trained-AFB₁.

Fig. 3. Effect of endurance exercise training for 14 weeks on the serum globulin and hepatic glutathione content measured 24 h after treatment with AFB₁ (2 mg/kg BW) in rats. Values are means $\pm$ SEM of 8–10 animals. Significant values are indicated by asterisks: ** $p < 0.01$, *** $p < 0.001$, significant difference between untrained-AFB₁ and trained-AFB₁.

their activities in untrained rats. In addition, potentiation of AFB₁ hepatotoxicity in trained rats was also supported by microscopic examination (data not shown), when the level of hepatotoxicity was compared between trained and untrained rats.

Serum globulin and hepatic glutathione content in rats given swimming training for 14 weeks and then treated with a single i.p. dose of AFB₁ are shown in Fig. 3. Serum globulin in untrained rats treated with AFB₁ was significantly greater ($p < 0.001$) than that in untrained rats treated with DMSO; whereas in trained rats, serum globulin was slightly decreased in those treated with AFB₁ when compared with the value for those treated with DMSO. At 24 h after AFB₁ treatment, serum globulin in trained rats was significantly decreased by 38.6% ($p < 0.01$) when compared with the level in untrained rats. The hepatic glutathione content significantly decreased in both trained and untrained rats treated with AFB₁ when compared with that for rats treated with DMSO. At 24 h after AFB₁ treatment, hepatic glutathione content in trained rats was 34.9% below that of untrained rats ($p < 0.001$). Serum globulin and hepatic glutathione content, however, were not significantly different between trained and untrained rats treated with DMSO.

DISCUSSION

An endurance training program involving 14 weeks of swimming activity induced significant physiological adaptation through a decrease in resting heart rate, an increase in heart weight, a decrease in body weight, and an increase in the activities of succinate dehydrogenase and citrate synthase (Table 1). Water immersion alone did not induce any adaptation of these parameters. Resting bradycardia

is a well-established phenomenon in endurance trained animals and humans [13, 14]. The present study showed a 13.5% decrease in resting heart rate in endurance-trained rats. The mechanism responsible for this phenomenon could have been alteration of the resting autonomic balance with an augmented parasympathetic dominance caused by slightly less sympathetic influence [14]. In addition, cardiac hypertrophy may have had a direct mechanical effect on the pacemaking mechanism. The biochemical adaptation that occurs in the limb muscles subjected to endurance training, i.e., a rise in the level of mitochondrial enzymes, produces an increase in the capacity for aerobic metabolism [15, 16]. The activities of succinate dehydrogenase and citrate synthase in this study were increased by 49.6 and 137%, respectively, in endurance-trained rats. A similar increase in enzyme activity was shown to have resulted from an increase in both size and number of mitochondria in the skeletal muscles of rats that had adapted to endurance exercise [17]. The endurance training program in the present study therefore provided sufficient stress to induce physiological adaptation, which indicates high physical fitness.

Endurance training did not affect protein synthesis, triglyceride synthesis and lipoprotein secretion in the liver, as indicated by the lack of change in the levels of serum albumin and globulin or in hepatic triglyceride content (Table 2). It is well known that the aminotransferase enzymes in blood are released from hepatic cells as a consequence of an increase in membrane permeability as well as due to membrane breakage. Serum AST and ALT activities were not altered by the training program in this study; hence the endurance exercise training did not disturb liver cell integrity.

Endurance training also did not affect the hepatic lipid peroxide level (Table 2). Since the rate of lipid peroxide generation depends on oxidative stress during exercise, lipid peroxidation that occurred in the membrane would disturb membrane integrity. Serum AST and ALT activities and the histopathological condition of the liver were not changed in endurance-trained rats. Therefore, it is possible that 14 weeks of swimming training was capable of inducing adaptive changes resulting in less accumulation of lipid peroxide during swimming. This assumption is supported by the findings of Alessio and Goldfarb [18].

The effect of chronic endurance exercise training on the activities of hepatic drug-metabolizing enzymes is still controversial. In the present study, endurance training increased the activities of hepatic microsomal aniline hydroxylase and *p*-nitroanisole-O-demethylase by 111 and 95.4%, respectively (Fig. 1). These results contrast with the findings of Ramos *et al.* [19] in which chronic swimming training did not increase the activity of *p*-nitroanisole-O-demethylase in Fischer rats. This discrepancy might have been due to the lower intensity of swimming in that study and perhaps to differences between the rat strains. No effect of endurance training on the activity of hepatic cytosolic glutathione-S-transferase was found in the present study. The hepatic glutathione content in this study was also not changed by endurance exercise training. This is in agreement with the results of Ohno *et al.* [20], which showed that chronic swimming can maintain the

hepatic glutathione content in equilibrium, whereas acute swimming or running can decrease it. This means that a high capacity for glutathione synthesis is produced by chronic endurance training. These evidences suggest that endurance exercise training can increase the activities of hepatic microsomal enzymes, especially in phase-I metabolism, as observed in this study.

Endurance exercise training enhanced the hepatocellular damage induced by AFB₁ as indicated by an increase in the activities of serum ALT (6.6 fold) and AST (1.8 fold) (Fig. 2) and by heightened severity of liver necrosis in this study. The mechanism responsible for the potentiation of AFB₁ hepatotoxicity by endurance training is not quite understood. However, we demonstrated that endurance training caused an increase in the activities of hepatic microsomal aniline hydroxylase and *p*-nitroanisole-O-demethylase (Fig. 1) without any changes in glutathione content at the time of AFB₁ administration (Table 2). Since aniline hydroxylase and *p*-nitroanisole-O-demethylase are representative mixed-function oxidase enzymes, it is probable that AFB₁ requires metabolic activation to exert its toxicity and that it is metabolized to the most reactive metabolite, AFB₁-8,9-oxide, by microsomal mixed-function oxidases in the liver. The covalent binding of AFB₁-8,9-oxide to hepatic nucleic acids, especially DNA, occurs in parallel with the toxigenic potency of AFB₁ [21]. Therefore, AFB₁-8,9-oxide formed through elevation of the activity of microsomal mixed-function oxidase enzymes is available for binding to hepatic macromolecules, thereby causing an increase AFB₁ hepatotoxicity during endurance exercise training.

In addition to reactive AFB₁-8,9-oxide, lipid peroxide is another reactive product that contributes to the hepatotoxicity induced by AFB₁. It has been reported that AFB₁-8,9-oxide possibly induces lipid peroxidation in hepatic subcellular fractions [22, 23]. However, we found that in endurance exercise-trained rats AFB₁ did not alter the lipid peroxide level in the liver, whereas in untrained rats the level of hepatic lipid peroxide induced by AFB₁ was increased 4.2 fold (Fig. 2). The mechanism by which endurance exercise training did not alter the hepatic lipid peroxide level induced by AFB₁ is still not known at present. Due to the decrease in hepatic glutathione level (Fig. 3) in the endurance exercise training group after AFB₁ treatment in comparison with the level in the untrained and AFB₁-treated group. This can be explained by the slight increase in the activity of hepatic cytosolic glutathione-S-transferase (Table 2) in the endurance exercise training group at the time of AFB₁ administration. Therefore, an increase in the hepatic cytosolic glutathione-S-transferase activity would lead to an increase in the amount of conjugation product of glutathione and AFB₁.

The 1.4 fold increase in serum globulin in the untrained and AFB₁-treated group (Fig. 3) was due to the leakage of globulin into the blood from the liver cells damaged by AFB₁. The serum globulin in the endurance exercise training and AFB₁-treated group was not changed. This might have been due to an increase in the amount of AFB₁-8,9-oxide induced by AFB₁ in the trained group, causing inhibition of hepatic globulin synthesis. The level of serum globulin is mostly

dependent on net synthesis rather than on simple leakage of globulin [24].

In conclusion, endurance exercise training enhanced the hepatotoxicity induced by AFB₁, as evidenced by a marked increase in serum AST and ALT activities. This was likely due to an increase in mixed-function oxidase enzyme activity at the time of AFB₁ administration during endurance exercise training.

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