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EFFECTS OF PIPERINE ON BIOENERGETIC FUNCTIONS OF ISOLATED RAT LIVER MITOCHONDRIA*

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Abstract—The *in vitro* effects of piperine on three bioenergetic reactions namely, oxidative phosphorylation, ATPase activity and calcium transport by isolated rat liver mitochondria have been investigated. Piperine was found to inhibit state 3 and DNP-stimulated respiration by mitochondria respiring with glutamate plus malate or succinate as substrates. The I_{50} values of piperine on oxidative phosphorylation in the presence of glutamate plus malate and succinate were 22 and 12 μg/mg mitochondrial protein respectively. With HTM preparations, the oxidation of added NADH and succinate was depressed by piperine while ascorbate plus TMPD oxidation was slightly affected. Piperine did not inhibit the mitochondrial ATPase activity induced by DNP, but by itself exerted stimulating activity on this enzyme. Piperine was also found to diminish calcium uptake and to facilitate the release of accumulated calcium by the mitochondria incubated with succinate or ATP. These results suggest that piperine inhibits mitochondrial oxidative phosphorylation at the level of respiratory chain, and the inhibitory site(s) is in the segment(s) ahead of cytochrome C. The mechanism of the piperine-induced ATPase activity is not known; but the effect of piperine on calcium transport is likely to be consequential to the effects of this compound on the mitochondrial respiratory chain and ATPase activity.

Piperine (1-piperoyl piperidine) is the principal alkaloidal constituent of black and long pepper, which are among the most common spices consumed by a large number of people worldwide. This compound is known to possess several pharmacological actions. Among its diverse pharmacological activities are antifertility effect [1], CNS depression and anti-inflammation [2], and inhibition of hepatic drug metabolism [3]. In addition, piperine has recently been demonstrated to inhibit growth and aflatoxin production of *Aspergillus parasiticus* in a dose-dependent manner [4]. Despite its many biological effects, the biochemical mechanisms of piperine actions are not well understood. It has been reported that piperine stimulates brain serotonin synthesis [5] but depletes substance P in the spinal cord [6]. As several inhibitors of mitochondrial energy metabolism are toxic to the fungi [7], the anti-fungal activity of piperine therefore suggests the possibility of mitochondrial effects by this compound. The present *in vitro* study shows that piperine inhibits oxidative phosphorylation and calcium accumulation, but stimulates ATPase activity of isolated rat liver mitochondria.

MATERIALS AND METHODS

Chemicals. All chemicals used were purchased from Sigma Chemical Co. Piperine was dissolved in absolute ethanol and only small volume (10 μl or less) was added to the reaction mixture; controls received equal volume of vehicle only.

Preparation of rat liver mitochondria. Male Wistar rats weighing 250–300 g were used throughout. Intact rat liver mitochondria were prepared by the method of Hogeboom [8]. The final suspension was in 0.25 M sucrose.

Hypotonic-treated rat liver mitochondria (HTM) were prepared by adding 3 ml of double-distilled water to 2 ml of freshly isolated mitochondrial suspension (approximately 60–80 mg protein). The resulting suspension was left at room temperature, with occasional stirring, for 1½ to 2 hr. At the end of this period, the preparation was transferred to an ice-bath and immediately used in the experiments.

The protein concentrations of all mitochondrial preparations were determined by the method of Lowry *et al.* [9] as modified by Miller [10] using bovine serum albumin as standard.

Oxygen consumption measurements. The oxygen uptake by intact mitochondria and HTM was determined by Clark oxygen electrode connected to an oxygen monitor (YSI model 53) and recorded on a strip-chart recorder (Gilson model N2).

ATPase activity. The mitochondrial ATPase activity was measured by determining the amount of inorganic phosphate liberated at the end of incubation period. The reaction was terminated by adding 1 ml aliquot of reaction mixture to 1 ml of ice-cold 20% trichloroacetic acid. After centrifugation, the amount of inorganic phosphate in the supernatant was determined by the method of Fiske and Subbarow [11].

* Abbreviations used: DNP, 2,4-dinitrophenol; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; HTM, hypotonic-treated mitochondria; NADH, reduced nicotinamide adenine dinucleotide; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

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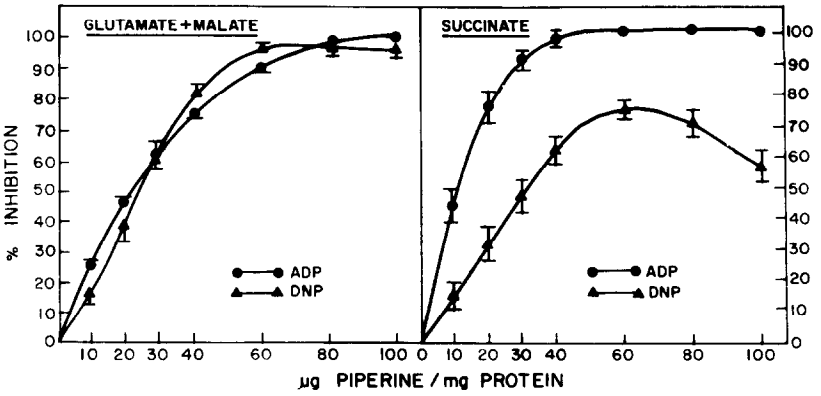

Fig. 1. The dose–response curves of piperine inhibition on the mitochondrial respiratory response to ADP and DNP with glutamate plus malate or succinate as substrates. Composition of reaction system: 38.15 mM Hepes buffer pH 7.4, 7.63 mM $MgCl_2$, 79.16 mM KCl, 1.91 mM potassium phosphate, 0.27 mM ADP, 0.05 mM DNP, 6.81 mM sucrose and piperine as indicated. When present, 5.45 mM potassium glutamate, 5.45 mM potassium malate and 5.45 mM succinate. ADP was added 1 min after piperine and DNP added during state 4 respiration. Total volume 1.84 ml. Each point represents a mean ± SEM from nine experiments. The control state 3 and uncoupled respiratory rates with glutamate plus malate and succinate as substrates were 232 ± 16 and 246 ± 12, and 260 ± 21 and 266 ± 15 ng-atoms 0/min/mg protein respectively.

Calcium transport measurements. The movement of calcium ions across mitochondrial membrane was followed by calcium-selective electrode (Orion model 93-20) and reference electrode (Orion model 90-02). The electrodes were connected to a pH/ion meter (Pope model 1502) and recorded on a strip-chart recorder (Gilson model N2).

Experimental conditions. All experiments were performed at 37° except calcium transport experiments in which the incubation was carried out in a small beaker at room temperature. The composition of reaction mixtures and other experimental conditions are described in the figure and table legends. The isolated mitochondria used in the present study must have the RCI value of at least four with glutamate plus malate as substrates. The experimental results reported here were reproducible with at least three separate mitochondrial preparations.

RESULTS AND DISCUSSION

Effects on intact mitochondria and HTM

Figure 1 reports the inhibition curves of piperine on the ADP- and DNP-stimulated respiration by intact rat liver mitochondria respiring with glutamate plus malate or succinate. When respiration was supported by NAD-linked substrate, piperine produced comparable inhibition on both state 3 and uncoupled respiration as the inhibition curves were nearly superimposed. The I_{50} values were 22 and 25 µg/mg mitochondrial protein for the ADP- and DNP-induced respiratory stimulation respectively. However when succinate was present as substrate, the uncoupled respiration induced by DNP was considerably less sensitive to piperine than state 3 respiration. The inhibition curve rose rather slowly; the maximum depression was about 75% and decreased with higher doses of piperine. The I_{50} values for state 3 and uncoupled respiration were 12 and 32 µg/mg protein respectively. It can also be seen from the titration curves that complete inhibition of mitochondrial oxidative phosphorylation occurred at approximately 100 and 50 µg/mg protein with glutamate plus malate and succinate respectively. Thus oxidative phosphorylation was about twofold more sensitive to piperine when supported by succinate than by NAD-linked substrates. The reasons for these differential sensitivity are not known at present. For comparison information, we have previously reported the I_{50} of capsaicin, a major pungent ingredient in hot pepper, on oxidative phosphorylation by rat liver mitochondria to be 16 µg/mg protein in the presence of glutamate as substrate [12]. Thus on the basis of I_{50} values piperine is somewhat less active than capsaicin on mitochondrial oxidative phosphorylation supported by NAD-linked substrates.

The inhibitory effects of piperine on both state 3 and uncoupled respiration suggested that this compound depressed oxidative phosphorylation through effect on the respiratory chain. The effect of piperine on electron transfer in different segments of the respiratory chain was studied with HTM supplemented with cytochrome *c* and different electron donors. The HTM preparation is a convenient system to study electron transport activity since these disrupted mitochondria lose respiratory control and oxidize substrates without phosphate acceptor. These HTM were completely uncoupled as indicated by the inability of 0.05–0.10 mM DNP to further stimulate respiration with any substrate tested. As reported in Table 1, piperine produced marked depression of HTM respiration supported by NADH and succinate but had little effect when ascorbate plus TMPD were electron donors. With 200 µg piperine, NADH and succinate oxidation were inhibited more than 65% whereas less than 10% inhibition was observed when ascorbate plus TMPD were substrates. Since the electrons from ascorbate plus TMPD enter respiratory chain at cytochrome *c* [13],

Table 1. Effect of piperine on HTM respiration with different substrates

Experiments	Respiration rates (ng-atoms 0/min/mg protein)		
	NADH	Succinate	Ascorbate + TMPD
Control	243 ± 22	164 ± 19	546 ± 56
100 μg piperine	113 ± 7	95 ± 11	524 ± 48
200 μg piperine	77 ± 5	53 ± 5	511 ± 45

Composition of reaction system: 18.18 mM Hepes buffer pH 7.4, 3.64 mM $MgCl_2$, 38.17 mM KCl, 8.02 mM sucrose, 100 μg cytochrome *c* and piperine as indicated. When present, 3.0 μmoles NADH, 5.34 mM succinate, 5.34 mM ascorbate and 0.53 mM TMPD. The mitochondria were preincubated with piperine for 1 min before substrates were added. The rates of oxygen uptake denote the differences between the rates after and before substrates addition. Values are means ± SEM from six experiments. Total volume 1.87 ml.

these results therefore indicated that the main site of piperine action on mitochondrial respiratory chain was somewhere in the region ahead of cytochrome *c*.

Effect on mitochondrial ATPase

The above results suggested the respiratory chain as the site of piperine inhibition but did not exclude the possibility that this compound might also depress oxidative phosphorylation by interfering with ADP phosphorylation. This possibility can be assessed by studying the effect of piperine on mitochondrial ATPase activity induced by uncoupler. The ATPase reaction is generally believed to represent the reversed process of the respiratory chain-linked ADP phosphorylation [14]. Agents which inhibit ATP synthesis, for example oligomycin [15], also depress the uncoupler-induced ATPase activity. The effect of piperine on mitochondrial ATPase activity with and without DNP was depicted in Fig. 2. DNP produced the usual ATPase stimulation with peak effect around 100 μM. When 150 μg piperine was also present, the enzyme activity appeared to be further enhanced at low uncoupler concentration. Some inhibition, which was insignificant statistically, was observed at high DNP concentrations where the uncoupler had maximum effect on the enzyme activity (left panel). This observation suggested that piperine might be able to activate the enzyme by itself. Indeed, further experiments with DNP omitted revealed the capacity of piperine to activate ATPase activity in a dose-related manner (right panel). DNP was evidently more active than piperine since the piperine-induced ATPase activity rose

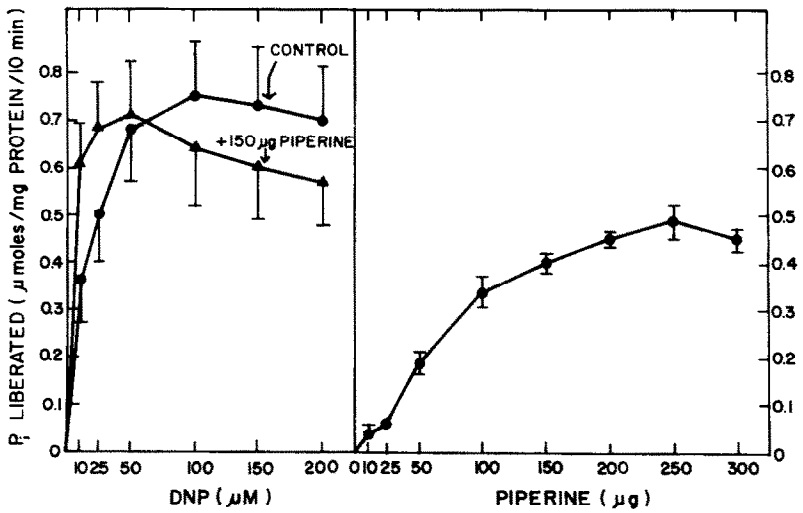

Fig. 2. Effect of piperine on the mitochondrial ATPase activity in the presence and absence of DNP. Composition of reaction system: 35.29 mM Hepes buffer pH 7.4, 7.06 mM $MgCl_2$, 73.22 mM KCl, 5.05 mM ATP, 16.84 mM sucrose, piperine and DNP as indicated. In the experiments in the left panel, the mitochondria were preincubated with DNP for 1 min before the addition of ATP, and piperine, when present, was added 1 min before DNP. In the right panel, the mitochondria were preincubated with piperine for 1 min before ATP was added. The reaction mixtures were further incubated for 10 min after ATP addition. Total volume 2.97 ml. Each point represents a mean ± SEM from six experiments.

slowly with less peak effect. Thus these results make it unlikely for piperine to have additional inhibitory effect on mitochondrial ADP phosphorylation.

The mechanism of the piperine-stimulated ATPase activity is unknown at present. The possibility that piperine may act by uncoupling the mitochondria cannot be totally excluded although this compound was found to cause no respiratory stimulation on mitochondria incubated with glutamate plus malate or succinate. This is because the respiratory stimulation consequential to uncoupling could be masked by the inhibitory effect of piperine on the respiratory chain. Other experiments revealed that, in analogy with the uncoupler-stimulated ATPase, the enzyme activity induced by piperine was also oligomycin-sensitive. In the presence of 10 μg oligomycin, the ATPase activity induced by 250 μg piperine was inhibited 43.8 ± 4.1% (mean ± SEM from twelve experiments).

Effect on mitochondrial calcium transport

It is well known that accumulation and retention of calcium ions by mitochondria require energy from substrate oxidation or ATP hydrolysis. When the process is powered by substrate oxidation, it is sensitive to respiratory chain inhibitors. Oligomycin and related compounds block the process supported by ATP hydrolysis [16]. Piperine was expected to affect mitochondrial calcium transport because of its apparent action on the respiratory chain. Preliminary study showed that 200 μg piperine severely inhibited the mitochondrial respiratory response to $CaCl_2 + P_i$ with glutamate plus malate or succinate as substrates. Further experiments were then performed using calcium-selective electrode with succinate or ATP as energy sources. The results are recorded in Fig. 3. In controls (curve A), calcium was rapidly taken up by the mitochondria and the accumulated calcium was well retained during 10 min incubation period. When 300 μg piperine was initially present in the medium, calcium uptake was diminished and the accumulated calcium was completely released subsequently (curve B). Similar calcium-releasing effect was also observed when piperine was added after the mitochondria were allowed to take up calcium for 2 min (curve C). It was consistently observed that piperine had a pronounced inhibitory effect on calcium uptake supported by succinate, in consonant with the effect on oxidative phosphorylation. The inhibitory effect on ATP-dependent calcium uptake was presumably related to the ATPase-stimulating action of piperine which would lower ATP level and reduce ATP availability for calcium transport. This notion was supported by other experiments not shown here in which addition of more ATP, after the piperine-induced calcium release was completed, caused the mitochondria to reaccumulate the released calcium. It should be pointed out that in these experiments relatively small ATP concentration (about 3 mM) was initially present in the incubation medium.

The present communication has demonstrated the effect of piperine on mitochondrial bioenergetics. Oxidative phosphorylation and calcium transport are

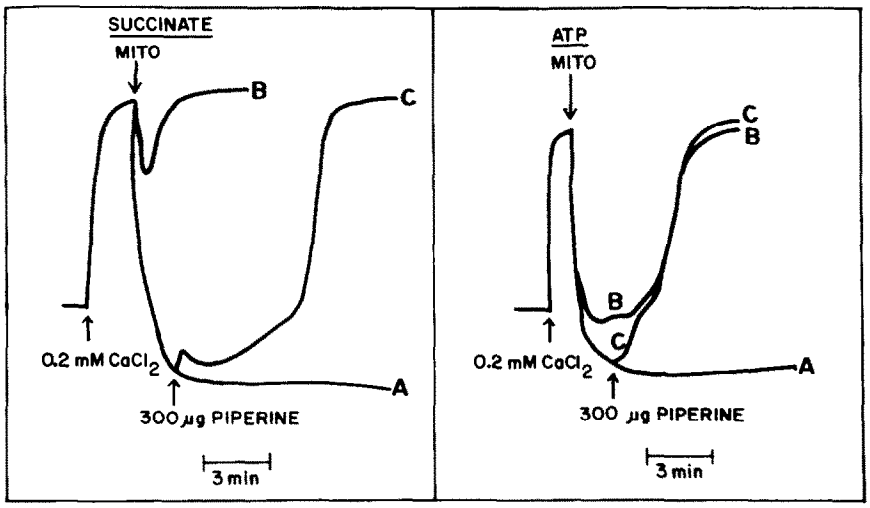

Fig. 3. Left panel: Effect of piperine on succinate-supported mitochondrial calcium transport. Composition of reaction system: 38.15 mM Hepes buffer pH 7.4, 1.91 mM $MgCl_2$, 87.75 mM KCl, 0.48 mM potassium phosphate, 5 mM potassium succinate, 10.22 mM sucrose, $CaCl_2$ and piperine as indicated. 1.88 mg mitochondrial protein per ml. Total volume 7.34 ml. Right panel: Effect of piperine on ATP-supported mitochondrial calcium transport. Composition of reaction system: 38.41 mM Hepes buffer pH 7.4, 1.92 mM $MgCl_2$, 88.35 mM KCl, 2.86 mM ATP, 8.57 mM sucrose, $CaCl_2$ and piperine as indicated. 1.29 mg mitochondrial protein per ml. Total volume 7.29 ml. In both panels: A, controls; B, 300 μg piperine was present initially in the incubation medium; C, 300 μg piperine added 2 min after the mitochondria. $CaCl_2$ was first added to calibrate the calcium-selective electrode. The distance of the upward deflection following $CaCl_2$ addition denotes the concentration in the medium of added calcium ion, i.e. 0.2 mM. Calcium transport was initiated by adding the mitochondria. The upward and downward deflections indicate the increase and decrease of calcium ion concentration in the reaction mixtures respectively.

inhibited while ATPase is stimulated by piperine. Experiments with HTM suggest that the site of piperine inhibition on the respiratory chain should span the segment ubiquinone to cytochrome *c* since piperine produces comparable depression on both NADH and succinate oxidation but has little effect when ascorbate plus TMPD are electron donors. However, two observations with intact mitochondria seem contradictory to this mechanism. Firstly, oxidative phosphorylation is about twofold more sensitive to piperine with succinate than with NAD-linked substrates. Secondly, when respiration is supported by succinate, the DNP-stimulated respiration is conspicuously less sensitive to piperine than state 3 respiration (Fig. 1). These discrepancies can be reconciled if it is assumed that piperine has two independent sites of action on the respiratory chain. The first site is the span NADH to ubiquinone and the other spans succinate to ubiquinone. The latter site being more reactive to piperine, and DNP can partially antagonize the inhibitory effect of piperine at this site. Preliminary experiments using CCCP as an uncoupler also show that piperine is more effective in depressing state 3 than uncoupled respiration with succinate as substrate. Thus the DNP-type uncouplers appear to interfere in some unknown way with the action of piperine at the second inhibition site (the span succinate to ubiquinone). It is noteworthy that the mitochondrial actions of piperine and capsaicin are different; the latter depresses the DNP-activated ATPase [12] and inhibits electron flow almost selectively at the span NADH to ubiquinone [17].

The acute and subacute toxicity of piperine in several animal species have been reported by Piyachaturawat *et al.* [18]. In mice, the LD_{50} values for single intravenous and intragastric administration are 15.1 and 330 mg/kg body wt respectively. This large differential toxicity presumably reflects poor gastrointestinal absorption and/or metabolic deactivation. If these data are applicable to humans, it explains why no serious adverse effect is encountered with pepper consumption. The average daily piperine ingestion in man, for example among Indian people [19], is very much less than the corresponding LD_{50} value in mice. It is not known to what extent the mitochondrial actions of piperine contribute to its many pharmacological activities. The amounts of piperine employed in the present study are comparable to those used by Madhyastha and Bhat [4] to demonstrate the antifungal activity. As many antimicrobials and antifungals are known to affect mitochondrial energy metabolism, the inhibitory effect of piperine on electron transport described here may intimately involve in the antifungal activity of this compound.

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