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MECHANISM OF THE INHIBITORY ACTION OF CAPSAICIN ON ENERGY METABOLISM BY RAT LIVER MITOCHONDRIA*

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Abstract—Capsaicin was found to inhibit the oxidation of exogenous NADH by digitonin particles and hypotonic-treated rat liver mitochondria without impairing the oxidation of succinate by these mitochondrial preparations. The action of methyl capsaicin, the non-phenolic derivative of capsaicin, on mitochondrial oxidative phosphorylation and NADH oxidation was found identical to that of capsaicin. However, on the basis of I_{50} values, methyl capsaicin was approximately three times more potent. The inhibition of NADH oxidation produced by capsaicin and its methylated derivative was completely relieved by addition of menadione, an electron acceptor at the NADH dehydrogenase segment of mitochondrial electron transport chain. From these observations, it is concluded that (a) both capsaicin and methyl capsaicin inhibit mitochondrial energy metabolism by retarding electrons flow from NADH to coenzyme Q; (b) the location of this action is beyond the site at which menadione accepts electrons from the NADH dehydrogenase system; and (c) the phenolic group in capsaicin molecule is not essential for the above mitochondrial action of this compound.

Capsaicin (trans-8-methyl-*N*-vanillyl-6-nonenamide) is a monophenolic and pungent compound in hot green and red pepper (capsicum fruits), the most common spices used in food preparations throughout the world. Capsaicin is a stable substance which persists in dried pepper with apparently unreduced potency and is freely soluble in hot water [1]. This compound has been employed as a pharmacological tool in the study of thermoregulation. Injection of capsaicin into the preoptic area of the rats produces a dose-dependent fall in body temperature and abolishes shivering [2]. However, with repeated injections, the hypothermic effect gradually diminishes and finally disappears. Thus capsaicin, after initial stimulation, desensitizes the hypothalamic temperature regulating system. This phenomenon is associated with the characteristic alterations of mitochondrial structure in certain preoptic neurons, which leads to the conclusion that these neurons are the thermodetector in the rats [3]. More recently, mitochondrial and other subcellular damages have been observed in the rat duodenal absorptive cells following intragastric or intraduodenal administration of capsaicin [4].

The mitochondrial alterations reported above have been revealed by means of electronmicrographs which raises the question whether capsaicin acts directly on the mitochondrial systems. In this regard, our recent *in vitro* study clearly demonstrates direct toxic effect of capsaicin on mitochondrial energy conservation [5]. Low concentrations of this compound inhibit the response of rat liver mitochondria, respiring with NAD-linked substrates, to the additions of ADP + P_i, DNP, and $CaCl_2$ + P_i. However, when the mitochondria are respiring in the presence of succinate, capsaicin is much less effective in depressing state 3 and uncoupled respiration. These findings suggest that capsaicin most probably interferes with mitochondrial energy transfer reactions at the level of electron transport chain; and, at low doses, this action is selectively confined to the first energy-conserving site. The present communication reports the effect of capsaicin on NADH and succinate oxidation by hypotonic-treated rat liver mitochondria and digitonin particles. In addition, the role of phenolic group in the capsaicin-induced inhibition of mitochondrial oxidative phosphorylation and NADH oxidation was assessed by comparing the mitochondrial effect of capsaicin with that of methyl capsaicin, the methyl ether derivative lacking the phenolic character.

MATERIALS AND METHODS

Chemicals. HEPES, DNP and NADH were purchased from Calbiochem. Capsaicin, digitonin, bovine serum albumin, menadione (2-methyl-1,4-naphthoquinone) and rotenone were obtained from Sigma Chemical Co. Methyl capsaicin was prepared from capsaicin by the method described by Lapworth and Royle [6]. It was purified by multiple crystallization from dilute methanol and dried in a vacuum desiccator. The identity of methyl capsaicin was confirmed by melting point and n.m.r. spectroscopy.

Preparations of intact rat liver mitochondria and mitochondrial fragments. Intact mitochondria from rat liver were prepared by the method of Hogeboom

* Abbreviations used: DNP, 2,4-dinitrophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HTM, hypotonic-treated rat liver mitochondria; NADH, reduced nicotinamide adenine dinucleotide; NAD, oxidized nicotinamide adenine dinucleotide.

[7] as described by Myers and Slater [8]. The final suspension was in 0.25 M sucrose.

Hypotonic-treated rat liver mitochondria (HTM) were prepared by suspending liver mitochondria from one rat (approximately 60 mg protein) in 2 ml of 0.1 M sucrose. The suspension was left at room temperature, with occasional stirring, for 2 hr. At the end of this period, the resulting suspension was transferred to an ice-bath and immediately used in the experiments.

The digitonin particles from rat liver mitochondria were prepared according to Vignais *et al.* [9]. The mitochondrial fragments obtained were suspended in distilled water and kept in an ice-bath. It was immediately used in the experiments.

The protein concentrations of all mitochondrial preparations were determined by the method of Lowry *et al.* [10] as modified by Miller [11] using bovine serum albumin as standard.

Measurements of mitochondrial oxygen consumption. The oxygen uptake by all mitochondrial preparations was measured in the chamber of a Gilson oxygraph equipped with a Clark oxygen electrode. The chamber was surrounded by a circulating water bath with temperature setting at 26°. The rates of oxygen consumption under various experimental conditions were expressed as μatom O/ml per min.

Determination of the NADH oxidized by mitochondrial fragments. In the experiments involving direct estimation of the amounts of NADH oxidized by HTM or digitonin particles, spectrophotometric method was employed. The reactions were terminated, after pre-determined period of incubation, by Millipore filtration. The amounts of NADH remained in the filtrates were calculated from the measured optical densities at 340 nm. The differences between added NADH and that left in the filtrates are the NADH oxidized by the mitochondrial fragments.

RESULTS

Effect of capsaicin on NADH and succinate oxidation by HTM and digitonin particles

It is well known that NADH poorly penetrates the membranes of intact mitochondria; however, after treatment of the mitochondria with hypotonic salt or sucrose solution the membrane permeability to NADH is increased [12]. The effect of capsaicin on NADH and succinate oxidation by hypotonic-treated rat liver mitochondria (HTM) is shown in Fig. 1. Curves A and D are the control responses of HTM to the additions of NADH and succinate respectively. Since both NADH and succinate stimulated oxygen consumption in the absence of phosphate acceptor, the oxidation of NADH and succinate by HTM apparently proceeded without phosphorylation. Preincubation of HTM with 300 μg capsaicin markedly depressed the ability of HTM to oxidize NADH, as evident by a much slower respiratory rate after NADH was added, while succinate oxidation was almost unaffected (curve B). The action of capsaicin was rapid in onset since addition of this compound during the NADH-induced respiratory stimulation caused immediate depression of the increased respiratory rate (curve C). These observations strongly suggest electrons transfer from NADH to coenzyme Q as the site of action of capsaicin. The inhibition curve of capsaicin on NADH-stimulated respiration by HTM is depicted in Fig. 2. The I_{50} was 10 μg/mg protein, a value considerably smaller than the reported I_{50} of 16 μg/mg protein for oxidative phosphorylation by intact mitochondria [5]. However, HTM was more resistant to inhibition when capsaicin was present in high doses. From Figure 1, 300 μg capsaicin (83 μg/mg protein) produced 75 per cent depression of NADH respiration by HTM (cf. Fig. 2) whereas the same dose can completely abolish state 3 respiration with intact preparation [5]. This

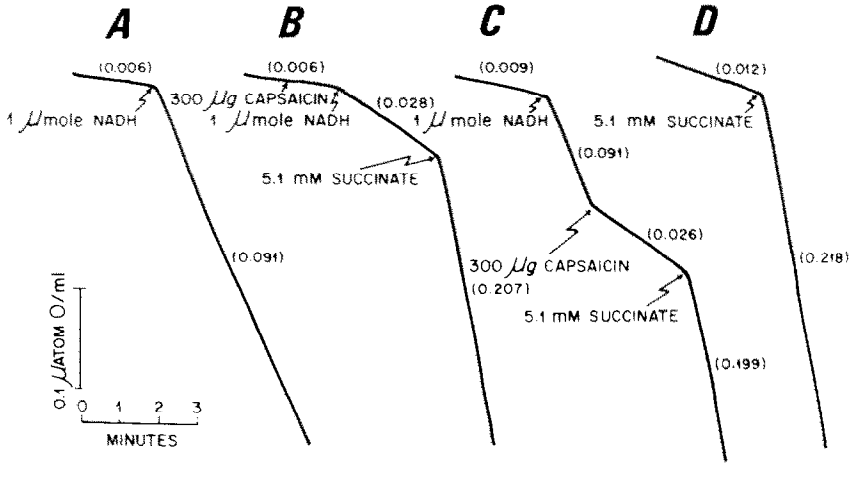

Fig. 1. Effect of capsaicin on NADH and succinate oxidation by hypotonic-treated rat liver mitochondria. Composition of reaction system: 16.8 mM HEPES buffer pH 7.4, 4.2 mM $MgCl_2$, 33.6 mM KCl, 12.7 mM sucrose. Capsaicin, NADH and succinate as indicated. 1.83 mg mitochondrial protein per ml. Total volume 1.96 ml. The figures in parentheses are rates of oxygen consumption in μatom O/ml per min.

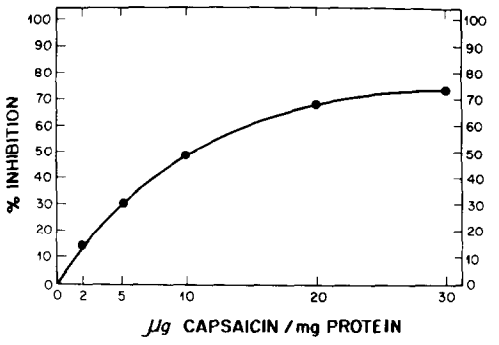

Fig. 2. The dose-response curve of capsaicin inhibition on NADH-induced respiration by hypotonic-treated rat liver mitochondria. Composition of reaction system: 17.5 mM HEPES buffer pH 7.4, 4.4 mM $MgCl_2$, 35.0 mM KCl, 8.0 mM sucrose, 1 μmole NADH, and capsaicin as indicated. NADH was added 1 min after capsaicin. Total volume 1.86 ml.

differential sensitivity presumably due to membrane perturbation following hypotonic treatment of the mitochondria.

Figure 3 demonstrates the effect of 300 μg capsaicin on NADH and succinate oxidation by digitonin particles. The results were essentially identical with those obtained with HTM. Because digitonin particles are devoid of phosphorylating activity [9], these findings provide additional support that capsaicin most probably act at site I electron transport chain.

Effect of methyl capsaicin on mitochondrial oxidative phosphorylation and NADH oxidation

Capsaicin is a monophenolic compound in which the phenolic group can be methylated to form methyl capsaicin, the derivative with much less pungency [6]. Many phenols are known to possess antibacterial activity; and this property has been ascribed to the ability of these chemicals to denature or inactivate cellular proteins and enzymes [13]. Thus, capsaicin may influence mitochondrial energy metabolism by interacting with mitochondrial enzyme systems because of its phenolic character. In order to assess the role of phenolic group in the mitochondrial action of capsaicin, the effect of methyl capsaicin on oxidative phosphorylation by intact rat liver mitochondria and on the oxidation of NADH by HTM was investigated. Figure 4 compares the actions of capsaicin and methyl capsaicin, both at 50 μg, on state 3 and uncoupled respiration by rat liver mitochondria. In these experiments glutamate and malate were respiratory substrates. Curve A shows the control response of the mitochondria to ADP and DNP. Capsaicin caused substantial depression of both state 3 and uncoupled respiration (curve B). However, almost complete block of mitochondrial oxidative phosphorylation was observed in the presence of methyl capsaicin (curve C). In other experiments not reported in Fig. 3, it was found that 50 μg methyl capsaicin produced less than 10 per cent inhibition of both ADP- and DNP-stimulated respiration when succinate was present as substrate.

The dose-response curve of the methyl capsaicin-induced inhibition on ADP- and DNP-stimulated respiration is recorded in Fig. 5. The degree of inhibition rose rapidly with small doses and leveled off when the doses exceeded 10 μg/mg protein. From these experiments, the I_{50} of methyl capsaicin on oxidative phosphorylation was 5 μg/mg protein. The corresponding value for capsaicin reported before was 16 μg/mg protein [5]. Thus on the basis of I_{50} values, methyl capsaicin was approximately three times more potent than capsaicin on mitochondrial oxidative phosphorylation. It was observed in other experiments not shown here that bovine serum albumin can effectively antagonize the effect of methyl capsaicin on state 3 respiration. Addition of 20 mg albumin caused more than 65 per cent reversal of the inhibited state 3 respiration produced by 80 μg methyl capsaicin. Similar antagonistic

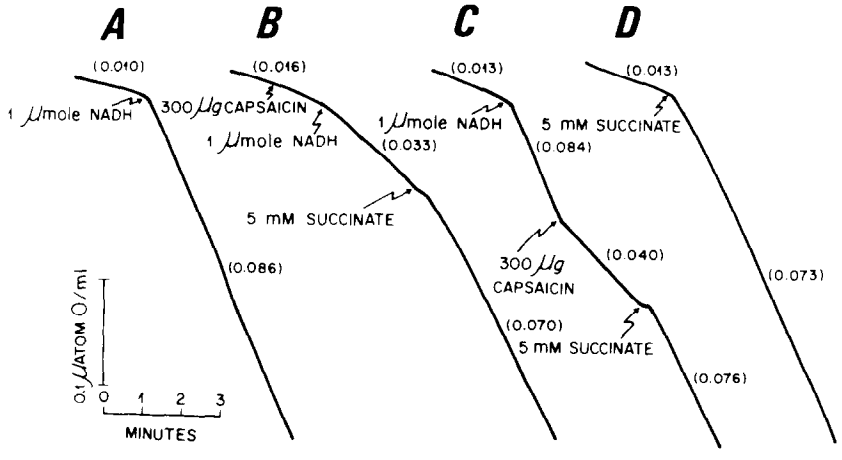

Fig. 3. Effect of capsaicin on NADH and succinate oxidation by digitonin particles. Composition of reaction system: 34.6 mM HEPES buffer pH 7.4, 8.7 mM $MgCl_2$, 69.5 mM KCl, capsaicin, NADH and succinate as indicated. 0.78 mg mitochondrial protein per ml. Total volume 1.96 ml. The figures in parentheses are rates of oxygen consumption in μatom O/ml per min.

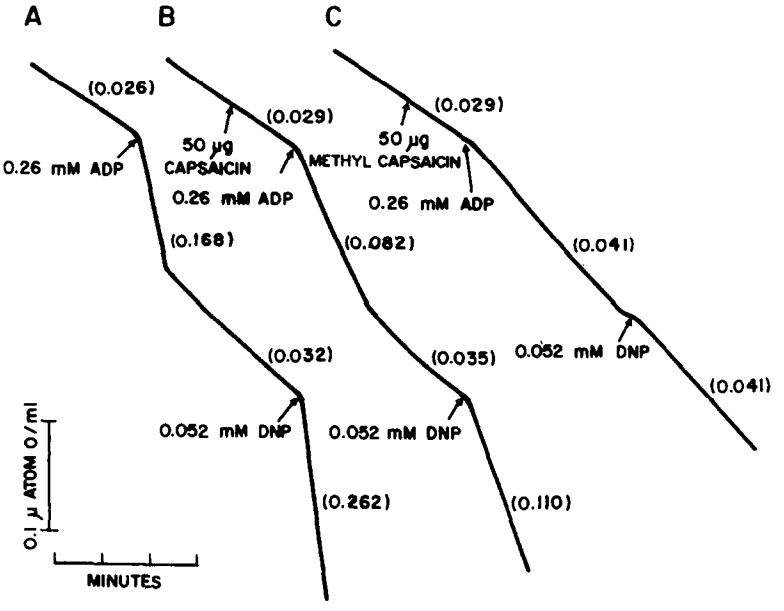

Fig. 4. Comparison of the inhibition induced by capsaicin and methyl capsaicin on state 3 and DNP-stimulated respiration by rat liver mitochondria. Composition of the reaction system: 31.3 mM HEPES buffer pH 7.4, 7.9 mM $MgCl_2$, 63.2 mM KCl, 19.7 mM sucrose, 2.6 mM potassium glutamate, 5.2 mM potassium malate, 2.6 mM potassium phosphate. ADP, DNP, capsaicin and methyl capsaicin as indicated. 2.2 mg mitochondrial protein per ml. Total volume 1.90 ml. The figures in parentheses are rates of oxygen consumption in μatom O/ml per min.

action of bovine serum albumin has already been described in the case of capsaicin [5].

Figure 6 shows the effect of methyl capsaicin on NADH and succinate oxidation by HTM. It is seen that methyl capsaicin acted rapidly to depress NADH oxidation without interfering with the oxidation of succinate. The I_{50} of methyl capsaicin on NADH oxidation by HTM as determined from the titration curve not reported here was found to be 3 μg/mg protein. The corresponding I_{50} of capsaicin mentioned above was 10 μg/mg protein. Thus, methyl capsaicin was about threefold more active than capsaicin on both oxidative phosphorylation by intact mitochondria and NADH oxidation by HTM.

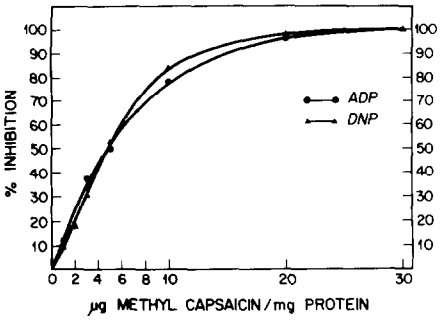

Fig. 5. Titration curve of the inhibitory effect of methyl capsaicin on the rate of respiratory response of rat liver mitochondria to ADP and DNP. Composition of reaction system: 31.2 mM HEPES buffer pH 7.4, 7.9 mM $MgCl_2$, 63.2 mM KCl, 19.7 mM sucrose, 7.9 mM potassium glutamate, 2.6 mM potassium phosphate, 0.26 mM ADP, 0.05 mM DNP, and methyl capsaicin as indicated. ADP was added after 1 min preincubation of the mitochondria with methyl capsaicin, and DNP added during state 4 respiration. Total volume 1.90 ml. Each point represents a mean from two separate experiments.

Reversal by menadione of the inhibitory actions of capsaicin and methyl capsaicin on NADH oxidation by HTM and digitonin particles

The effects of rotenone, methyl capsaicin and capsaicin on NADH oxidation by HTM, and the relief of these effects by menadione is recorded in Table 1. In these experiments NADH oxidation was measured directly by spectrophotometric method as described in Materials and Methods. Rotenone, one of the most potent inhibitors of mitochondrial NADH dehydrogenase known [14], at 10 μg severely retarded NADH oxidation. 100 μg methyl capsaicin caused comparable inhibition while 100 μg capsaicin was considerable less effective presumably because of its lower potency. Additions of menadione, a naphthoquinone derivative which accepts electrons from the NADH dehydrogenase [15], completely relieved the inhibitory actions of rotenone, methyl capsaicin and capsaicin. Similar results were also obtained with digitonin particles. It should be pointed out that small quantity (10 μg) of menadione was effective in these experiments because menadiol, the reduction product of menadione, can be reoxidized via coenzyme Q and cytochrome systems [16].

DISCUSSION

We have previously reported the toxic action of low doses of capsaicin on oxidative phosphorylation by rat liver mitochondria respiring in the presence of NAD-linked substrates but not with succinate [5].

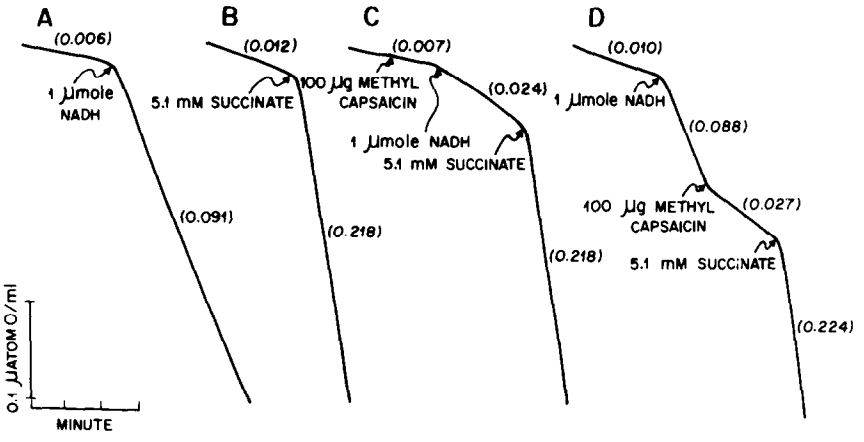

Fig. 6. Effect of methyl capsaicin on NADH and succinate oxidation by hypotonic-treated rat liver mitochondria. The composition of reaction system is identical to that reported in Fig. 1 except methyl capsaicin was present instead of capsaicin. The figures in parentheses are rates of oxygen consumption in μatom O/ml per min.

In this paper we directly demonstrate the inhibition of NADH oxidation by capsaicin with two mitochondrial preparations, the HTM and digitonin particles. The results clearly show the capability of capsaicin to retard NADH oxidation while succinate oxidation is almost unaffected. Because the HTM and digitonin particles [9] used in the present study oxidize substrates without coupled phosphorylation, these observations strongly indicate inhibition of electrons transfer from NADH to coenzyme Q (site I) as the mechanism of capsaicin action. It is well known that NADH dehydrogenase is the enzyme complex mediating the movement of electrons from NADH to coenzyme Q [17]. The complexity of this enzyme can be partly appreciated by the ability of ferricyanide and various quinones to accept electrons from different regions of the enzyme system [15]. The reversal by menadione of the capsaicin-induced inhibition of NADH oxidation indicates that the site of capsaicin blockage is beyond the site at which menadione interacts with the NADH dehydrogenase system. Whether capsaicin impedes electrons transfer by direct action on the NADH dehydrogenase is not known at present. However, the possibility of capsaicin acting by combining with sulfhydryl groups present in this enzyme [18] seems unlikely since dithiothreitol, a sulfhydryl-protecting reagent [19], was found totally inert against the action of capsaicin on mitochondrial oxidative phosphorylation [5].

Comparison of the effects of capsaicin and methyl capsaicin on mitochondrial oxidative phosphorylation and NADH oxidation reveals identical pattern of actions, suggesting similar mechanism is involved. Of particular interest is the observed greater potency of the methyl ether derivative over the parent compound. Thus, methylation of the phenolic group in capsaicin molecule potentiates its mitochondrial activity. It is, therefore, apparent that the phenolic function is not required for the action of capsaicin on the mitochondria. In a comparative study on the

Table 1. Effect of menadione on the inhibitory actions of rotenone, methyl capsaicin and capsaicin on NADH oxidation by hypotonic-treated rat liver mitochondria

Experiments	NADH oxidized (μmoles/2 min)
Control	0.68
10 μg rotenone	0.23
10 μg rotenone + 10 μg menadione	0.79
100 μg methyl capsaicin	0.28
100 μg methyl capsaicin + 10 μg menadione	0.77
100 μg capsaicin	0.47
100 μg capsaicin + 10 μg menadione	0.77

Composition of reaction system: 18.5 mM HEPES buffer pH 7.4, 4.4 mM $MgCl_2$, 37 mM KCl, 5.0 mM sucrose, 0.87 μmoles NADH, rotenone, methyl capsaicin, capsaicin and menadione as indicated. NADH was added 1 min after the inhibitors. Menadione, when present, was added simultaneously with the inhibitors. After additions of NADH, the reaction mixtures were further incubated for 2 min before Millipore filtration. The amounts of NADH oxidized were calculated as described in Materials and Methods. 1.33 mg mitochondrial protein per ml. Total volume 3.0 ml.

sensory effects of capsaicin and its congeners, Szolcsányi and Jancsó-Gábor [20, 21] found that the acylamide linkage and alkyl chain are not required for the pain-producing activity and pungency of these chemicals, but the presence of phenolic group is necessary. In contrast, the desensitizing effect has been found to depend on the acylamide structure since its replacement by esteric group causes total loss of this activity. It is also known that the phenomenon of capsaicin desensitization is associated with mitochondrial structural damages in certain spinal and preoptic neurons [22, 3]. The mitochondrial changes observed in these neurons may, at least partly, result from direct toxic action of capsaicin on the mitochondria [5]. Thus, these studies correlate the acylamide structure with the desensitizing and mitochondrial activities of capsaicin. Our results are in agreement with the above findings since they indicate that the phenolic structure is unessential for the mitochondrial action of capsaicin, which implicate the involvement of the acylamide linkage. At present, it is not clear why methyl capsaicin is more active than capsaicin on mitochondrial energy metabolism, but an increase in lipid solubility is a possibility. The greater potency of methyl capsaicin warrants further investigations on the metabolic fate of capsaicin inasmuch as hot pepper is widely used in food preparations. It should be noted in this connection that hepatic cirrhosis [23] and a reduction in growth rate [24] have been observed in animals receiving diets supplemented with hot pepper or capsaicin.

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