Pergamon

Biochemical Pharmacology, Vol. 50, No. 6, pp. 885–888, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00198-0

Short Communication

EFFECTS OF ANANDAMIDE ON HEPATIC FATTY ACID METABOLISM

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(*Received* 14 *December* 1994; *accepted* 25 *April* 1995)

Abstract—Incubation of rat hepatocytes with anandamide (arachidonoylethanolamide) inhibited acetyl-CoA carboxylase activity and fatty acid synthesis *de novo* without affecting fatty acid synthase. This was concomitant to a decrease in the intracellular levels of malonyl-CoA. Likewise, anandamide depressed both cholesterol synthesis *de novo* and the incorporation of exogenous palmitate into triacylglycerols and phospholipids. On the other hand, anandamide stimulated in parallel both carnitine palmitoyltransferase I activity and ketogenesis from palmitate, though ketogenesis from octanoate was unaffected. The effects of anandamide on hepatic fatty acid synthesis and oxidation were: (a) mimicked by arachidonic acid, a product of anandamide breakdown by anandamide amidase; (b) prevented by phenylmethylsulfonyl fluoride, an inhibitor of anandamide amidase; and (c) not affected by bisindolylmaleimide, a specific inhibitor of protein kinase C. Furthermore, δ^9-tetrahydrocannabinol had no effect on any of the parameters determined, ruling out the possibility that the effects of anandamide on hepatic fatty acid metabolism are mediated by the peripheral cannabinoid receptor. The results thus indicate that anandamide might function as a carrier of arachidonic acid in the modulation of hepatic fatty metabolism.

Key words: anandamide; δ^9-tetrahydrocannabinol; arachidonic acid; acetyl-CoA carboxylase; carnitine palmitoyltransferase I; hepatocyte

An arachidonic acid derivative (arachidonoylethanolamide, generally termed "anandamide") has recently been isolated from porcine brain [1] and identified as an endogenous ligand of cannabinoid receptors [1–3]. Anandamide formation occurs in the brain through phosphodiesterase-mediated cleavage of N-arachidonoyl-phosphatidylethanolamine [4], although the CoA- and ATP-independent synthesis of anandamide from arachidonic acid and ethanolamine has been reported as well [5]. The effects of anandamide are similar to those elicited by psychotropic cannabinoids, especially THC§ (reviewed in ref. 6, 7). Mechoulam and coworkers have also reported the existence of other N-amide derivatives of long-chain unsaturated fatty acids that exert THC-like actions [7]. These observations indicate that the cannabinoid receptor ligand is actually a family of endogenous compounds with similar chemical features [7].

In spite of their relatively simple structural characteristics, long-chain fatty acids play an important role in the regulation of elementary biological processes such as the immune response, the maintenance of membrane potential, and the mechanisms of receptor-mediated signal transduction [8–11]. In addition, fatty acids exert modulatory effects on different pathways of hepatic lipid metabolism [8, 12]. Since anandamide contains an arachidonoyl moiety [1] that might play a role in the modulation of hepatic fatty acid metabolism, the present work was undertaken to study in detail the effects of anandamide on the different fatty acid-metabolizing pathways in rat hepatocytes, as well as the possible mechanism(s) involved in these potential effects of anandamide.

Materials and Methods

Isolation and incubation of hepatocytes. Male Wistar rats (250–300 g) that had free access to food and water were used throughout this study. Hepatocytes were isolated and incubated as described in ref. 13.

Rates of fatty acid metabolism. The rate of fatty acid synthesis *de novo* was monitored by the incorporation of ^3H_2O (1.0 Ci/l in the final incubation) into total fatty acids [13]. Tritium incorporation into cholesterol was determined in the same incubations [14]. The rate of fatty acid esterification was determined by the incorporation of [U-^{14}C]palmitate (0.5 Ci/mol, 0.4 mM final concentration) into cellular triacylglycerols and phospholipids [14]. The rate of fatty acid oxidation was determined by the formation of ketone bodies (which routinely accounted for 90–95% of total oxidation products) from [1-^{14}C]fatty acid (either palmitate or octanoate, 0.5 Ci/mol, 0.4 mM final concentration) [14].

Enzymatic assays. Enzyme activities were determined in digitonin-permeabilized hepatocytes. Acetyl-CoA carboxylase (ACC) activity was determined as the incorporation of [1-^{14}C]acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction [13]. Carnitine palmitoyltransferase I (CPT-I) activity was determined as the tetradecylglycidate-sensitive incorporation of L-[*Me*-^{14}C]carnitine into palmitoylcarnitine [15]. Fatty acid synthase activity was determined as the malonyl-CoA-dependent incorporation of [1^{14}C]acetyl-CoA into fatty acids [13].

Malonyl-CoA concentration. Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method [15].

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§ Abbreviations: ACC, acetyl-CoA carboxylase; CPT-I, carnitine palmitoyltransferase I (carnitine palmitoyltransferase located in the mitochondrial outer membrane); PMSF, phenylmethylsulfonyl fluoride; THC, δ^9-tetrahydrocannabinol.

Statistical analysis. Results shown represent the means ± SD of the number of animals indicated in every case. Cell incubations and/or enzyme assays were always carried out in triplicate. Statistical analysis was performed by the Student *t*-test.

Materials. Tetradecylglycidic acid and 2-chloro-6-phenylhexanoate were kindly donated by Dr. Math J. H. Geelen, Utrecht University (The Netherlands). Anandamide was from Cayman Chemical (Ann Arbor, MI, U.S.A.). THC was kindly donated by the National Institute of Drug Abuse (U.S.A.). Arachidonic acid and phenylmethylsulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bisindolylmaleimide was from Calbiochem (San Diego, CA, U.S.A.).

Results and Discussion

Effects of anandamide on hepatic fatty acid oxidation. The rate of ketogenesis from palmitate was increased by the addition of anandamide to the hepatocyte incubation medium (Fig. 1A). However, anandamide had no significant effect on the rate of ketogenesis from octanoate (Fig. 1A). Since palmitate is transported into mitochondria by a carnitine-dependent process, whereas octanoate may enter mitochondria independently of carnitine [16], the target for anandamide action might be CPT-I. As can be seen in Table 1, hepatic CPT-I activity was stimulated by the addition of anandamide to the cell incubation medium.

Anandamide has been shown to act on different enzyme systems and biological processes via receptor-dependent [7] and receptor-independent mechanisms [17, 18]. Addition of a supramaximal dose of THC to the hepatocyte incubation medium did not have any effect on either ketogenesis from palmitate or CPT-I activity (Table 1), indicating that the effect of anandamide on these two parameters is not mediated by its binding to a cannabinoid receptor. Likewise, no cannabinoid receptor has been detected to date in rat liver tissue [19]. When PMSF was added to the incubation medium to inhibit the anandamide-degrading amidase [20], the effect of anandamide on ketogenesis and CPT-I activity was not evident (Table 1). In contrast, arachidonic acid (alone or in the presence of PMSF) mimicked the stimulatory effect of anandamide on hepatic ketogenesis and CPT-I activity (Table 1). Hence, arachidonic acid released from the amidase-mediated anandamide breakdown would mediate the stimulation of hepatic fatty acid oxidation by anandamide. The stimulation of hepatic ketogenesis and CPT-I activity by anandamide or arachidonic acid was not antagonized by 2.0 mM 2-chloro-6-phenylhexanoate, an inhibitor of fatty acid β-oxidation (results not shown), indicating that metabolization of arachidonic acid is not necessary for the effects of anandamide to be demonstrated.

Like THC [21], arachidonic acid has been shown to activate protein kinase C [22], which in turn has been suggested to modulate hepatic ketogenesis and CPT-I activity [16]. Thus, hepatocytes were incubated with anandamide or arachidonic acid in the presence of bisindolylmaleimide, a potent and specific inhibitor of protein kinase C that antagonizes the phorbol ester-induced inhibition of hepatic CPT-I (M. Guzmán and G. Velasco, unpublished observation). However, the stimulation of hepatic ketogenesis and CPT-I activity by anandamide or arachidonic acid was not affected by bisindolylmaleimide (Table 1), ruling out any possible involvement of protein kinase C in these effects.

Effects of anandamide on hepatic lipogenesis. Incubation of hepatocytes with anandamide markedly decreased the rate of fatty acid synthesis *de novo* (Fig. 1B). This decrease correlated well with the anandamide-mediated inhibition of ACC activity (Table 1), whereas fatty acid synthase activity was not affected by the addition of 5 μM anandamide to the hepatocyte incubation medium (results not shown). Likewise, the intracellular concentration of malonyl-CoA, the product of the reaction catalyzed by ACC and a physiological inhibitor of CPT-I [16], was decreased in parallel by anandamide. Values of malonyl-CoA concentration (in nmol/mg of cell protein) were 0.075 ± 0.013 and 0.026 ± 0.004 for incubations with no additions and 5 μM anandamide, respectively ($n = 3$, $P < 0.01$). Since hepatic CPT-I and palmitate oxidation are stimulated in concert by anandamide (see above), this would indicate that the deinhibition of CPT-I resulting from the decrease in malonyl-CoA concentration may be the factor responsible for the regulation of hepatic long-chain fatty acid oxidation under these conditions. In fact, the binding of long-chain acyl-CoA (including arachidonoyl-

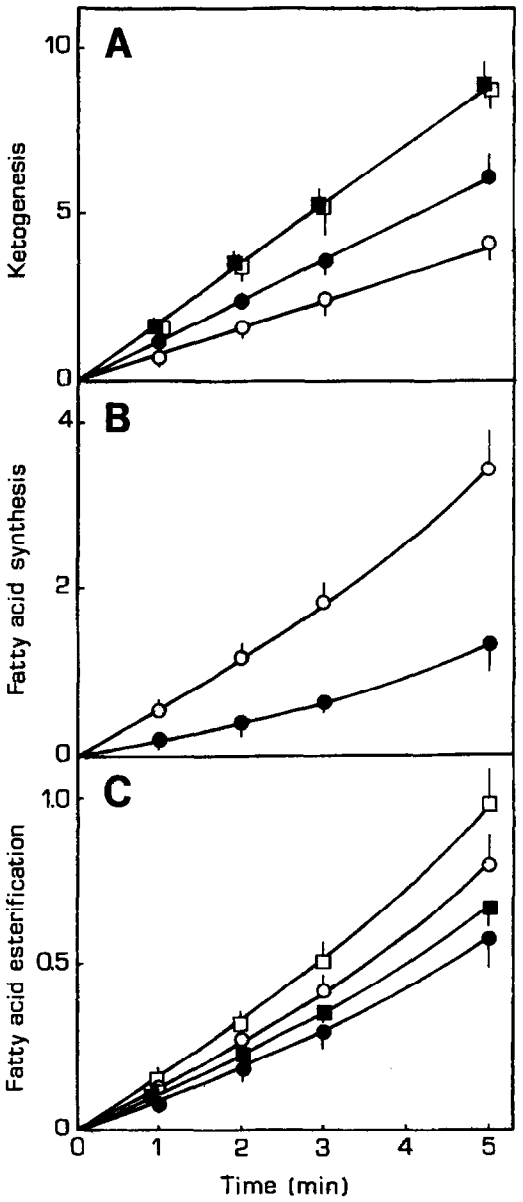

Fig. 1. Effects of anandamide on hepatic ketogenesis, fatty acid synthesis *de novo*, and fatty acid esterification. Hepatocytes were incubated for up to 5 min with the respective radiolabelled precursors either in the absence (open symbols) or in the presence of 5 μM anandamide (close symbols). Panel A: Ketogenesis was determined with either 0.4 mM [^{14}C]palmitate (circles) or 0.4 mM [^{14}C]octanoate (squares) as a substrate. Panel B: Fatty acid synthesis *de novo* was determined with $^{3}H_2O$ as a precursor. Panel C: Fatty acid esterification into triacylglycerols (circles) and phospholipids (squares) was determined with 0.4 mM [^{14}C]palmitate as a substrate. Results are expressed as nmol of precursor into product/mg of cell protein and correspond to three different animals. Note the scale on the *y* axis.

Table 1. Effects of anandamide, arachidonic acid, THC, PMSF, and bisindolylmaleimide on CPT-I activity, ketogenesis from palmitate, ACC activity, and fatty acid synthesis *de novo* in rat hepatocytes

Additions	CPT-I activity (%)	Rate of ketogenesis (%)	ACC activity (%)	Rate of fatty acid synthesis (%)
None	100	100	100	100
Anandamide (5 μM)	153 ± 6*	148 ± 8*	38 ± 6*	40 ± 8*
THC (20 μM)	103 ± 6	101 ± 5	101 ± 5	105 ± 8
PMSF (2 mM)	98 ± 5	96 ± 7	96 ± 7	99 ± 3
Anandamide (5 μM) + PMSF (2 mM)	103 ± 9	100 ± 7	93 ± 7	96 ± 5
Arachidonic acid (5 μM)	161 ± 11*	156 ± 9*	36 ± 7*	35 ± 9*
Arachidonic acid (5 μM) + PMSF (2 mM)	157 ± 10*	151 ± 13*	32 ± 3*	30 ± 5*
Bisindolylmaleimide (2 μM)	99 ± 4	105 ± 8	96 ± 4	95 ± 4
Anandamide (5 μM) + bisindolylmaleimide (2 μM)	150 ± 11*	153 ± 4*	32 ± 10*	33 ± 3*
Arachidonic acid (5 μM) + bisindolylmaleimide (2 μM)	149 ± 10*	153 ± 6*	37 ± 9*	35 ± 4*

Hepatocytes were incubated for either 2 min (determination of enzyme activities) or 5 min (determination of rates of fatty acid metabolism) in the presence of the additions indicated. One hundred percent values were as follows: CPT-I activity, 1.64 ± 0.21 nmol product/min per mg cell protein; ACC activity, 0.94 ± 0.13 nmol product/min per mg cell protein; ketogenesis, 49.4 ± 5.7 nmol palmitate into ketone bodies/h per mg cell protein; fatty acid synthesis *de novo*, 38.8 ± 4.3 nmol acetyl units/h per mg cell protein. Results correspond to 4 different animals.

* $P < 0.01$ vs incubations with no additions.

CoA) to ACC induces the depolimerization and subsequent inactivation of the enzyme (cf. ref. 13). Long-chain acyl-CoA have also been shown to stimulate the AMP-activated protein kinase kinase, which phosphorylates and activates the AMP-activated protein kinase [23]. This in turn phosphorylates and inactivates two key enzymes of hepatic lipid metabolism, viz. ACC and 3-hydroxy-3-methylglutaryl-CoA reductase [23]. In line with this observation, addition of 5 μM anandamide to the hepatocyte incubation medium inhibited cholesterol synthesis *de novo* by 53 ± 3% ($n = 3$, $P < 0.01$).

Like the stimulation of hepatic fatty acid oxidation, the anandamide-mediated inhibition of ACC and fatty acid synthesis *de novo* was: (a) mimicked by arachidonic acid; (b) prevented by PMSF; and (c) not affected by bisindolylmaleimide (Table 1). In addition, ACC activity and fatty acid synthesis *de novo* were not affected by the addition of THC to the incubation medium (Table 1). Hence, arachidonic acid released from the amidase-mediated anandamide breakdown would mediate the inhibition of hepatic fatty acid synthesis by anandamide.

With regard to fatty acid esterification, anandamide inhibited the incorporation of exogenous [^{14}C]palmitate into the two major hepatocellular lipid classes, namely, triacylglycerols and phospholipids (Fig. 1C). This effect of anandamide might be due to modulatory effects of arachidonic acid on enzymes involved in hepatic glycerolipid synthesis [12] and/or to a simple shift of [^{14}C]palmitate into oxidation (see above), thus removing substrate for esterification.

Conclusions. Our results show that anandamide exerts remarkable effects on hepatic fatty acid metabolism by inhibiting lipogenic processes and stimulating ketogenesis. They also indicate that these effects may be mediated by the arachidonic acid released from anandamide upon breakdown by anandamide amidase. A possible physiological role of anandamide in liver is indicated by the observations that rat liver homogenates are able to both degrade and synthesize anandamide [5, 20], and that mouse liver cytochrome P450 metabolizes anandamide to at least 10 different metabolites [24]. However, the significance of hepatic anandamide metabolism remains unexplored. In addition, anandamide (arachidonoylethanolamide) is not the only acylethanolamide characterized so far from animal tissues [7, 25]. Interestingly, it was 30 years ago that Bachur *et al.* reported the existence of significant amounts of palmitoylethanolamide in brain, liver, and skeletal muscle from fasted rats and guinea pigs [26]. The possible role of this acylethanolamide in cellular metabolism is still unknown, although we have observed that the effects of saturated long-chain fatty acids (e.g., palmitic, stearic) on hepatic lipid metabolism are rather less remarkable than the effects of unsaturated fatty acids (e.g., linoleic, arachidonic) (authors' unpublished observations). Our current research is directed toward the elucidation of the physiological meaning of the effects described herein.

Acknowledgements—This work was supported by grants from UCM (PR179/3527) and PFIZER. The authors are indebted to the National Institute of Drug Abuse (U.S.A.) for kindly providing THC.

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