Evidence that the AMP-activated protein kinase stimulates rat liver carnitine palmitoyltransferase I by phosphorylating cytoskeletal components

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Abstract The activity of hepatic carnitine palmitoyltransferase I (CPT-I) may be modulated by interactions with cytoskeletal components [Velasco et al. (1998) J. Biol. Chem. 273, 21497–21504]. We have studied whether the AMP-activated protein kinase (AMPK) is involved in this process. AMPK stimulated CPT-I in permeabilized hepatocytes but not in isolated liver mitochondria. In addition, AMPK abrogated the inhibition of CPT-I of isolated mitochondria induced by a cytoskeletal fraction. These two effects of AMPK were not evident when the kinase was inactivated by pretreatment with protein phosphatase 2C. Cytokeratins 8 and 18 were phosphorylated by AMPK in vitro and by incubation of intact hepatocytes with 5-aminoimidazole-4-carboxamide ribonucleoside, a cell-permeable activator of AMPK. These results provide the first evidence that AMPK stimulates CPT-I by direct phosphorylation of cytoskeletal components.

Key words: AMP-activated protein kinase; Carnitine palmitoyltransferase I; Cytoskeleton; Hepatocyte

1. Introduction

The AMP-activated protein kinase (AMPK) plays a major role in the cellular response to metabolic stress [1,2]. AMPK is activated by AMP and by phosphorylation by an upstream kinase, which is itself activated by AMP [2,3]. Once activated, AMPK phosphorylates and inactivates a number of regulatory enzymes involved in biosynthetic pathways, thereby preventing further ATP utilization when ATP depletion ensues in situations such as fuel limitation and hypoxia [2,4]. AMPK plays an important role in the control of lipid metabolism. Thus, AMPK phosphorylates and inactivates acetyl-CoA carboxylase (fatty acid synthesis), 3-hydroxy-3-methylglutaryl-CoA reductase (sterol/isoprenoid synthesis) and hormone-sensitive lipase (triacylglycerol/cholesteryl ester breakdown) [2]. Although several protein kinases can phosphorylate purified acetyl-CoA carboxylase in vitro, there is good evidence demonstrating that in intact hepatocytes and in the liver in vivo AMPK is the major protein kinase responsible for the inactivation of acetyl-CoA carboxylase by phosphorylation (cf. [2]). Modulation of acetyl-CoA carboxylase activity by AMPK is essential for the control of long-chain fatty acid oxidation since malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase, is the physiological inhibitor of carnitine palmitoyltransferase I (CPT-I), the key regulatory enzyme of long-chain fatty acid oxidation [5–7]. Activation of AMPK has been shown to decrease malonyl-CoA levels and to stimulate fatty acid oxidation in heart [8], skeletal muscle [9] and liver [10].

Although evidence has accumulated during the last two decades highlighting the physiological importance of malonyl-CoA inhibition of CPT-I [6,7], an additional mechanism of control of CPT-I activity has been put forward. Studies using permeabilized hepatocytes have shown that various agents exert short-term effects on CPT-I activity in parallel with changes in the rate of long-chain fatty acid oxidation [5,11]. These changes in CPT-I activity are assumed to be mediated by a malonyl-CoA-independent mechanism since they are very stable and survive complete removal of malonyl-CoA from the medium [12]. Recent observations indicate that this malonyl-CoA-independent control of hepatic CPT-I activity may rely on the modulation of interactions between mitochondria and cytoskeletal components, most likely intermediate filaments, so that disruption of the latter leads to de-inhibition of CPT-I [13–15].

We have observed that the incubation of hepatocytes with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a cell-permeable activator of AMPK, stimulates CPT-I, and this stimulation partially survives cell permeabilization and extensive washing of the permeabilized cells, pointing to a possible malonyl-CoA-independent process [10]. The present work was therefore undertaken to study the possible role of AMPK in the malonyl-CoA-independent control of CPT-I activity.

2. Materials and methods

2.1. Materials

AMPK was purified from rat liver by immunoprecipitation with specific antibodies bound to protein A-Sepharose [4]. Recombinant human protein phosphatase 2C [16] was a generous gift from Dr. R.K. Beri (Zeneca Pharmaceuticals, Macclesfield, UK). Tetradecylglycidate was kindly donated by Dr. J.M. Lowenstein (Brandeis University, Waltham, MA, USA). Purified cytokeratins 8 and 18 were from ICN Pharmaceuticals (Costa Mesa, CA, USA). The anti-pan cytokeratin monoclonal antibody was from Sigma (St. Louis, MO, USA).

2.2. Assay of CPT-I activity

CPT-I activity was determined in isolated mitochondria as the malonyl-CoA-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine exactly as described before [12]. When CPT-I activity was determined in suspensions of mitochondria containing a total-cytoskeleton fraction, the latter was isolated as described in

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Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CPT-I, carnitine palmitoyltransferase I

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[15]. In other experiments, CPT-I activity was determined in permeabilized hepatocytes as the tetradecylglycidate-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine as previously described [12]. Hepatocytes were permeabilized with digitonin and then extensively washed prior to determination of enzyme activity ('two-step assay' in [10,12]).

2.3. Incubations with AMPK

To study the effect of AMPK on CPT-I activity, isolated mitochondria or digitonin-permeabilized hepatocytes were suspended in buffer A, consisting of 50 mM Tris-HCl, pH 7.5, 50 mM NaF, 1 mM EDTA, 5 mM sodium pyrophosphate, 1 mM dithiothreitol and 10% (v/v) glycerol. This medium was supplemented with 0.2 mM AMP, 0.2 mM ATP and 5 mM $MgCl_2$. After the addition of purified AMPK, incubations were run for 10 min at 30°C and CPT-I activity was subsequently determined as described above. Reactions of AMPK dephosphorylation were performed by washing 5 times the AMPK-resin complex with buffer B (buffer A without the protein phosphatase inhibitors NaF and sodium pyrophosphate) and further incubation for 10 min at 30°C in buffer B with 5 mM $MgCl_2$ and 0.32 mg/ml protein phosphatase 2C. Parallel controls were run in which the phosphatase was omitted from the incubations. The resulting AMPK is designated as 'washed AMPK' in Figs. 1 and 2.

To study the phosphorylation of cytokeratins by AMPK, assays were run in buffer A together with purified AMPK, 0.2 mM AMP, 0.2 mM [γ-^{32}P]ATP (2 μCi/assay), 5 mM $MgCl_2$ and 0.2 mg/ml purified cytokeratin 8 or 18. Positive controls were run in the presence of 0.2 mM SAMS peptide, whereas negative controls were performed in the absence of AMPK or cytokeratins. After incubation for 15 min at 30°C, samples were resolved by SDS-PAGE as described in [4].

2.4. Determination of the stoichiometry of cytokeratin phosphorylation in intact hepatocytes

The stoichiometry of cytokeratin phosphorylation in intact hepatocytes was determined as described before [15] by simultaneously calculating (i) the specific radioactivity of the γ-phosphate of intracellular ATP after labelling of the cells with $^{32}P_i$ and separation of the adenine nucleotides by HPLC; (ii) the amount of ^{32}P incorporated into the cytokeratin bands after labelling of the cells with $^{32}P_i$ and immunoprecipitation/SDS-PAGE of the cytokeratins; and (iii) the mass of protein in those cytokeratin bands after immunoprecipitation of the cytokeratins from ^{32}P-free hepatocyte incubations and resolution by SDS-PAGE in parallel with different amounts of purified cytokeratins 8 and 18.

2.5. Statistical analysis

Results shown represent the means ± S.D. of the number of experiments indicated in each case. Incubations and enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t*-test.

3. Results and discussion

3.1. AMPK stimulates CPT-I in permeabilized hepatocytes but not in isolated liver mitochondria

Addition of purified AMPK to isolated liver mitochondria did not exert any significant effect on CPT-I activity (Fig. 1A). In contrast, AMPK stimulated CPT-I activity in digitonin-permeabilized hepatocytes by over 2-fold (Fig. 1B). The stimulatory effect of AMPK on CPT-I was not due to a non-specific effect of the anti-AMPK antibody-protein A-Sepharose complex, and was not evident when the kinase was pretreated with protein phosphatase 2C (Fig. 1B), a phosphatase that has been shown to dephosphorylate and inactivate AMPK [2,16]. These data indicate that extramitochondrial cell components are required for the regulation of CPT-I by AMPK. It has been shown previously that neither cyclic AMP-dependent protein kinase nor protein kinase C exerts any significant effect on CPT-I activity in permeabilized hepatocytes, whereas Ca^{2+}/calmodulin-dependent protein kinase II caused a modest activation of CPT-I (approximately 40%) [15]. Taken together, these results suggest that AMPK may play a prominent role in the malonyl-CoA-independent control of hepatic CPT-I activity.

3.2. AMPK abrogates the inhibitory effect of a cytoskeletal fraction on CPT-I

In order to define the cell components that are sufficient for the malonyl-CoA-independent control of CPT-I to occur, a simple reconstitution system was used consisting of isolated mitochondria together with a cytoskeletal fraction (cf. [15]). As shown in Fig. 2, the inhibition of CPT-I produced by exposure of isolated mitochondria to the cytoskeletal fraction was reverted by addition of exogenous AMPK. This effect was prevented by pretreatment of the kinase with protein phosphatase 2C (Fig. 2). Hence it is likely that AMPK

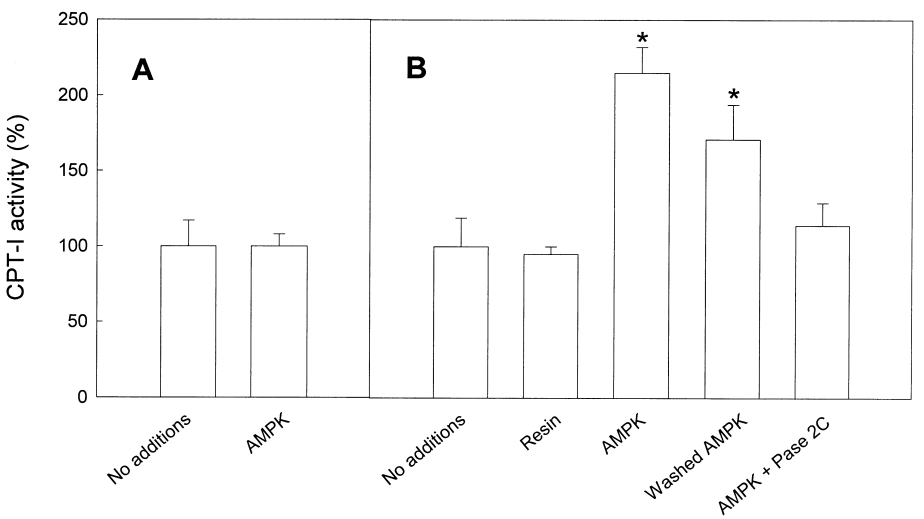

Fig. 1. AMPK stimulates CPT-I in permeabilized hepatocytes but not in isolated liver mitochondria. A: CPT-I activity in isolated liver mitochondria. B: CPT-I activity in permeabilized hepatocytes. 'Resin' denotes the anti-AMPK antibody-protein A-Sepharose complex. Results correspond to four different experiments. One hundred percent values of CPT-I activity (in nmol/min/mg protein) were 7.86 ± 1.34 in isolated mitochondria and 2.09 ± 0.40 in permeabilized hepatocytes. $*P < 0.01$ vs. incubations with no additions.

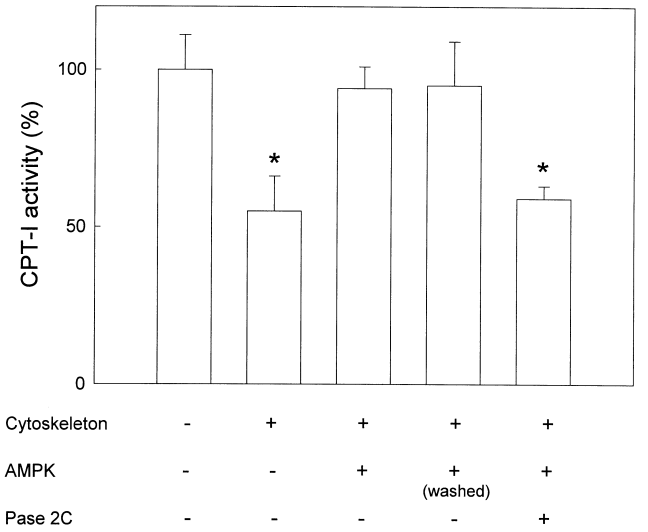

Fig. 2. AMPK abrogates the inhibitory effect of a cytoskeletal fraction on CPT-I. Isolated liver mitochondria (1.5–2.0 mg protein/ml) were preincubated for 10 min in the absence (−) or presence (+) of a cytoskeletal fraction (0.05–0.06 mg protein/ml). AMPK was subsequently added (+) or not (−) to the incubations. The kinase had been pretreated (+) or not (−) with protein phosphatase 2C. Results correspond to four different experiments. $*P < 0.01$ vs. incubations with no additions.

stimulates CPT-I by acting on certain cytoskeletal components.

3.3. AMPK phosphorylates cytokeratins 8 and 18 in vitro and in intact hepatocytes

We have recently shown that incubation of hepatocytes with colchicine (a microtubule disrupter) and cytochalasin (a microfilament disrupter) has no effect on CPT-I activity, whereas cell exposure to 3,3′-iminodipropionitrile (an intermediate filament disrupter) produces a significant stimulation of CPT-I [15]. This and other observations indicate that intermediate filaments may be the cytoskeletal components involved in the malonyl-CoA-independent modulation of CPT-I activity [15]. AMPK was unable to significantly (less than 0.05 mol phosphate per mol protein) phosphorylate purified tubulin (microtubules) and actin (microfilaments). In contrast, purified cytokeratins 8 and 18, the major components of liver intermediate filaments [17], were readily phosphorylated by AMPK in vitro (Fig. 3A). These results are consistent with the hypothesis that AMPK activates CPT-I in a malonyl-CoA independent manner by modification of the intermediate filament organization.

The phosphorylation pattern of purified cytokeratins in vitro may not reflect their phosphorylation status in a more physiological, whole-cell system (cf. [18,19]). Intact hepatocytes were labelled with $^{32}P_i$ and cytokeratins were immunoprecipitated after exposing the cells to AICAR. The protein phosphatase inhibitor okadaic acid was used as a positive control since it has been shown to induce cytokeratin hyperphosphorylation [15]. As shown in Fig. 3B, cytokeratins 8 and 18 were phosphorylated by okadaic acid and – to a lower extent – by AICAR. Since phosphorylation of cytokeratins 8 and 18 has been shown to induce the disruption of liver intermediate filaments [17–19], these data further support the hypothesis that AMPK may be involved in the control of cytoskeletal dynamics.

In both the in vitro (Fig. 3A) and the whole-cell experiments (Fig. 3B) the extent of phosphorylation of cytokeratin 8 was higher than that of cytokeratin 18. The stoichiometry of phosphorylation of the purified cytokeratins by AMPK in vitro (Fig. 3A) was significantly lower than that of the AICAR-induced phosphorylation of cytokeratins in intact hepatocytes (Fig. 3B). We do not know the reason for this difference although it may be due, at least in part, to the way in which the in vitro assays are performed. The solubility of cytokeratins in aqueous solutions is extremely low, and they tend to form filamentous complexes in vitro [17]. The AMPK used for the phosphorylation is present in an immune complex and this, combined with the low solubility of the cytokeratins, might prevent, or hinder, the kinase from phosphorylating some of the sites within the cytokeratins. Whatever the reason, it is clear that AMPK phosphorylates cytokeratins 8 and 18 in vitro and that AICAR mimics this effect in hepatocytes.

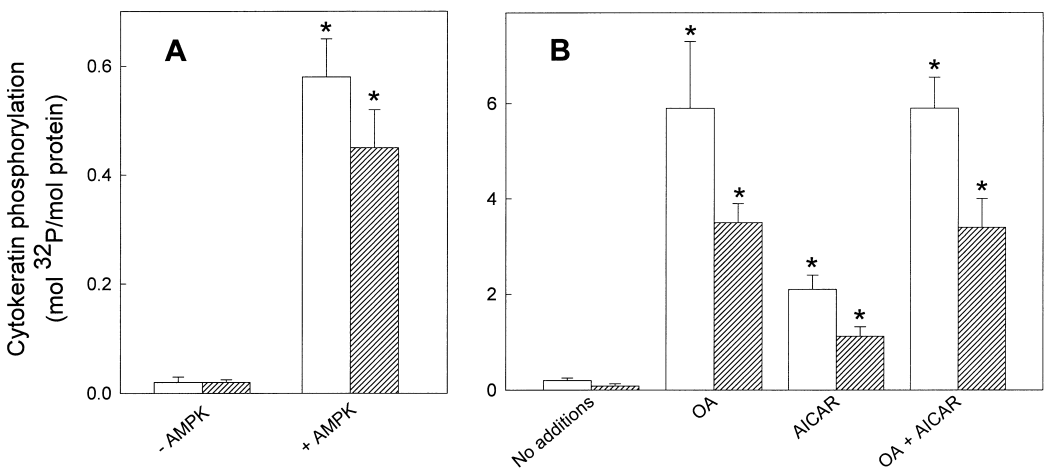

Fig. 3. AMPK phosphorylates cytokeratins 8 and 18 in vitro and in intact hepatocytes. A: Phosphorylation of purified cytokeratins 8 and 18 in vitro. B: Phosphorylation of cytokeratins 8 and 18 in intact hepatocytes. After labelling with $^{32}P_i$, hepatocytes were incubated for 15 min in the absence or presence of 0.5 mM AICAR and/or 0.5 μM okadaic acid (OA). Open bars: cytokeratin 8; hatched bars: cytokeratin 18. Note the different scales on the *y*-axes. Results correspond to four different experiments. $*P < 0.01$ vs. incubations with no additions.

3.4. Possible physiological implications

The physiological importance of the malonyl-CoA-independent control of hepatic CPT-I activity is still unclear. In vitro and in vivo experiments indicate that the dynamics of mitochondria in living cells may be modulated by specific interactions with the cytoskeleton [20,21]. Since the organization of intermediate filaments changes dramatically in a number of liver pathologies [21,22], it might be expected that CPT-I activity would be affected in parallel under these conditions. Indeed, this has been shown to occur in transformed liver cells [23]. Despite this, it is obvious that the existence of malonyl-CoA-independent regulation of CPT-I does not diminish the importance of malonyl-CoA as a physiological modulator of CPT-I activity [6,7]. It seems likely, however, that the malonyl-CoA-dependent and malonyl-CoA-independent control of hepatic CPT-I by AMPK may operate in concert ([10] and the present report).

Although it is widely accepted that AMPK plays a pivotal role in the regulation of energy metabolism [2], recent evidence indicates that this kinase may regulate a wider array of cellular functions such as gene expression [24,25], extracellular matrix-evoked cell growth [26] and cytoskeletal dynamics (the present report). Furthermore, the possibility that AMPK may control CPT-I activity by phosphorylating cytoskeletal components supports the emerging regulatory role of the cytoskeleton in intracellular signalling [21,22,27].

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