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Biochemical and Biophysical Research Communications 362 (2007) 206–211
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Activation of mammalian target of rapamycin complex 1 and insulin resistance induced by palmitate in hepatocytes

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Received 31 July 2007
Available online 9 August 2007

Abstract

Excessive supply of fatty acids to the liver might be a contributing factor to hepatic insulin resistance associated with obesity and type 2 diabetes mellitus. The aim of this study was to investigate direct effects of palmitate on insulin signaling in hepatocytes. The ability of metformin to reverse changes induced by palmitate was also studied. Rat hepatocytes in primary culture exhibited a rightward shift of the insulin dose–response curve for PKB phosphorylation during culture with palmitate. The insulin-stimulated phosphorylation of GSK-3β, a metabolic substrate of PKB, was diminished in palmitate hepatocytes. By contrast, the mTOR protein kinase was overstimulated in cells incubated with palmitate. Hepatocytes cultured with palmitate displayed hyperphosphorylation of IRS-1 at Ser residues 632/635, known to be phosphorylated by mTOR. Metformin treatment of the hepatocytes resulted in activation of the AMP-activated kinase, attenuation of the mTOR/S6K1 pathway, reduction of IRS-1 phosphorylation, and a leftward shift in the insulin dose–response curve for PKB activation. These data suggest a link between an oversupply of fatty acid to hepatocytes, a disproportionate stimulation of mTOR/S6K1, and resistance to insulin.

Keywords: Insulin signaling; Insulin resistance; Liver; Hepatocytes; PKB; Akt; mTOR; Fatty acid; Palmitate; Metformin

The liver plays a major role in glucose homeostasis, based on its capacity for net glucose uptake from the blood during hyperglycaemia, and net glucose release during postabsorptive and fasting periods [1]. The balance between hepatic glucose uptake and release depends mainly on the flux of substrates along the opposing pathways of glycogen synthesis/glycogenolysis and glycolysis/gluconeogenesis [2]. A key regulator of these pathways is insulin, acting through the covalent control of enzyme activities by phosphorylation or dephosphorylation [3] and through induction or repression of genes for metabolic enzymes [4].

Insulin resistance, particularly in the liver, is a critical feature of type 2 diabetes mellitus (T2DM) [5] and of conditions predisposing to T2DM such as obesity and the metabolic syndrome [6]. Although insulin resistance is a multifactorial disorder involving multiple mechanisms, a suspected cause of insulin resistance in the liver is the increased delivery of fatty acids to this tissue and the hepatocellular deposition of excess lipid, termed hepatosteatosis or non-alcoholic fatty liver disease [7,8]. Most investigations suggesting a connection between an hepatic overload of fatty acids and insulin resistance were conducted in whole animals after several days of high fat feeding or during systemic infusion of lipids, making it difficult to distinguish direct effects of excess lipid in the liver from indirect effects due to prolonged hyperglycaemia, hyperinsulinaemia, or extrahepatic effects of free fatty acids [7,9]. In the current study, we investigated direct effects of the prototype saturated fatty acid palmitic acid on insulin signaling in rat hepatocytes in primary culture. The focus was on key components of intracellular signaling cascades, including protein kinase B (PKB, also named Akt), mammalian target of rapamycin (mTOR), AMP-activated

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doi:10.1016/j.bbrc.2007.08.004

protein kinase (AMPK) and the insulin receptor substrate-1 (IRS-1). The ability of the antidiabetic drug metformin to reverse the effects of palmitate was also investigated.

Materials and methods

Materials. Cell culture materials, antibodies and other reagents were obtained from sources listed in Supplementary methods.

Isolation and culture of hepatocytes. Male Wistar rats weighing between 190 and 270 g were deprived of food 68 h before hepatocyte isolation and resupplied with standard chow 23 h before hepatocyte isolation. The drinking water was replaced by a glucose solution (20% w/v) for the last 17 h before the isolation of hepatocytes. The animals were anesthetized with thiopental and hepatocytes were isolated after collagenase perfusion of the livers and cultured using published procedures [4], with minor modifications. Details on the hepatocyte culture protocol are given in Supplementary methods.

Preparation of protein extracts and immunoblot assays. Lysis of hepatocyte monolayers and preparation of protein lysates were done essentially as described [10]. Measurement of protein concentration, SDS–PAGE and immunoblotting were by published methods [10,11]. Immunoblots were revealed by enhanced chemiluminescence using reagents from Pierce Biotechnology, and quantified by densitometer scanning of X-ray films as specified in Supplementary methods. Control for equal protein loads between lanes was performed by immunoblotting with antibodies to total PKB or GSK-3 which provided invariant signal in the different experimental conditions.

Results

Insulin resistance in hepatocytes cultured with palmitate and re-sensitization by metformin

Insulin triggers a rapid and sustained phosphorylation of S473 in PKB in primary rat hepatocytes, an effect accompanied by activation of the kinase [12,13]. The degree of PKB phosphorylation at S473 was measured in hepatocytes cultured in presence of 0.4 mM palmitate prior to stimulation with a range of insulin concentrations from 0.39 nM to the near-maximal concentration of 6.25 nM (Fig. 1A). Compared to control cells without fatty acid supplement, hepatocytes in the presence of palmitate exhibited a rightward shift in the insulin dose–response. Calculated EC_{50} values for insulin were 1.8 nM in control cells and 3.4 nM with palmitate, corresponding to a 47% decrease in insulin sensitivity.

Insulin stimulation of PKB phosphorylation was also measured in hepatocytes incubated with palmitate, followed by treatment with 1 mM metformin together with palmitate for the last 6 h of culture prior to insulin addition (Fig. 1A). The insulin log dose–response curve was strongly shifted to the left after metformin treatment of hepatocytes cultured with palmitate. The insulin EC_{50} in palmitate cells after metformin treatment was 1.3 nM, markedly lower than the EC_{50} of 3.4 nM in palmitate cells without metformin, and slightly below the EC_{50} of 1.8 nM in control cells incubated without palmitate. Thus, metformin hypersensitized hepatocytes to insulin during culture with palmitate.

Other signaling events along the phosphoinositide 3-kinase (PI3K)/PKB pathway were investigated in the three culture conditions described above (Fig. 1B). Phosphorylation of PKB at T308 as well as at S473 was examined. Phosphorylation at both sites is required for maximal kinase activity of PKB toward some but not all substrates [14]. In addition, the level of S9 phosphorylation in GSK-3β, a substrates of PKB, was monitored to provide an estimate of PKB activity prevailing in the cells. Insulin stimulation of control hepatocytes resulted in readily discernible phosphorylation of both S473 and T308 of PKB, as well as S9 of GSK-3β. The three effects were attenuated in hepatocytes cultured with palmitate and were restored to normal or supranormal levels by metformin treatment of palmitate cells (Fig. 1B).

Overactivation of the mTOR/S6K1 cascade in hepatocytes cultured with palmitate and reversal by metformin

The protein kinase mTOR, as part of a multiprotein assembly called mTOR complex 1 (mTORC1), represents a node in cell signaling, receiving positive input from PKB as well as from cell autonomous nutrient sensing systems. A key substrate of mTORC1 is the downstream 70 kDa ribosomal protein S6 kinase 1 (S6K1) which is specifically activated by mTOR-dependent phosphorylation at T389 [15]. Phosphorylation of S6K1 at T389 can therefore serve as an estimate of mTOR activity in mTORC1. In the absence of insulin stimulation, hepatocytes cultured in the presence of palmitate exhibited a 4-fold increase in T389 phosphorylation of S6K1 compared to cells in palmitate-free medium. The palmitate effect was strongly suppressed following treatment with metformin (Fig. 2A).

In hepatocytes cultured with palmitate and stimulated for 1 h with a dose of insulin close to the EC_{50}, the phosphorylation of PKB at S473 was reduced by approximately 50% compared to control cells (Fig. 2B). In contrast, T389 phosphorylation of S6K1 was slightly enhanced above the control level. Metformin treatment of the palmitate hepatocytes resulted in superinduction of insulin-dependent PKB phosphorylation to 180% of the level noted in control cells. At the same time, insulin-stimulated S6K1 phosphorylation was reduced 50% below the control level. Thus, culture of hepatocytes with palmitate was accompanied by spontaneous activation of the mTOR/S6K1 protein kinase cascade in the absence of insulin. After insulin stimulation, the mTOR/S6K1 sytem in hepatocytes cultured with palmitate was overstimulated in relation to the degree of PKB phosphorylation. Finally, metformin treatment of palmitate cells suppressed the overactivation of the mTOR/S6K1 system and enhanced the insulin-stimulated phosphorylation of PKB.

Hyperphosphorylation of IRS-1 at S632/S635 in hepatocytes cultured with palmitate

An important consequence of the activation of the mTOR/S6K1 protein kinase cascade in insulin signaling is the functional inhibition of the insulin receptor sub-

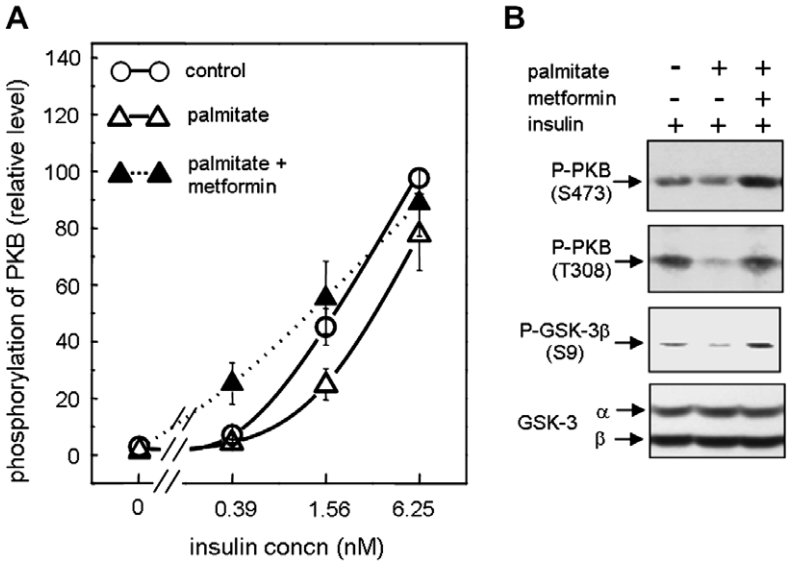

Fig. 1. Decreased insulin sensitivity in hepatocytes cultured with palmitate and re-sensitization by metformin treatment. (A) Dose–response curves of insulin-stimulated PKB phosphorylation (S473) in control hepatocytes (○), hepatocytes cultured with palmitate (△), or hepatocytes cultured with palmitate and treated with metformin (▲). Hepatocytes were exposed to palmitate (0.4 mM) for 22 h before insulin stimulation. Metformin treatment (1 mM) was done in the presence of palmitate during the last 6 h before insulin addition. Insulin was allowed to act for 1 h. Immunoblots from five independent hepatocyte experiments were subjected to quantification by densitometer scanning. Relative phosphorylation levels were expressed in percentage of the level in control hepatocytes stimulated with 6.25 nM insulin in each experiment. Data are means ± SEM. (B) Effects of palmitate and metformin on PKB-mediated insulin signaling in cultured hepatocytes. Protocol for culture with palmitate or palmitate plus metformin as in (A). Insulin treatment was with 1.56 nM insulin for 1 h before cell harvest. Immunoblot analysis was performed with phosphospecific antibodies against PKB and GSK-3β phosphorylated at the designated residues. Equal protein loading was verified using antibodies to total GSK-3. The experiment was repeated three times.

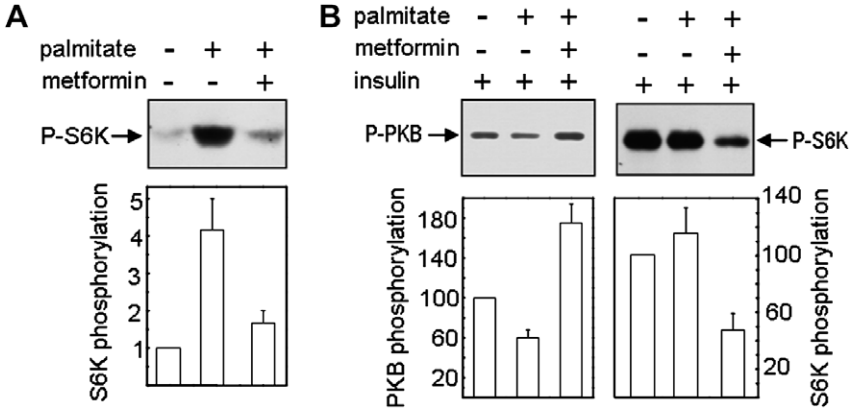

Fig. 2. Effect of palmitate without or with metformin on phosphorylation of S6K1 in the basal state (A) and on phosphorylation of PKB and S6K1 after insulin stimulation (B). (A) Basal level (no insulin) of T389 phosphorylation of S6K1 in control hepatocytes and in hepatocytes cultured with palmitate, without or with metformin treatment. Image of an immunoblot and plot summarizing the scanning data from three separate experiments are shown. Values were expressed as—fold increases (means ± SEM) compared to control hepatocytes. (B) Effects of insulin (1.56 nM for 1 h) on PKB (S473) phosphorylation (left-hand panels) and S6K1 (T389) phosphorylation (right-hand panels) in hepatocytes cultured under the indicated conditions. Quantitative data from four separate hepatocyte experiments, expressed as percentage of value in control hepatocytes, are given as means ± SEM. The film exposure time of the phopho-S6K1 blot in (A) was approximately 10 times longer than in (B).

strate-1 (IRS-1), following its site-specific phosphorylation at residues S632/S635 by mTOR [16,17] and S302 by S6K1 [18]. Total protein from hepatocytes during culture without or with palmitate was extracted in the basal state or 1 h after addition of 3.12 nM insulin to estimate the amount of IRS-1 and the degree of S632/S635 phosphorylation. Immunoblots of total IRS-1 showed that the cell content of IRS-1 was not reduced in hepatocytes cultured with palmitate. However, there was an electrophoretic mobility shift of a fraction of IRS-1, which was retarded from position denoted *L* to position *M* (Fig. 3A). In both control and palmitate hepatocytes, insulin caused a more pronounced retardation of IRS-1, which migrated in majority to position denoted *H* in Fig. 3.

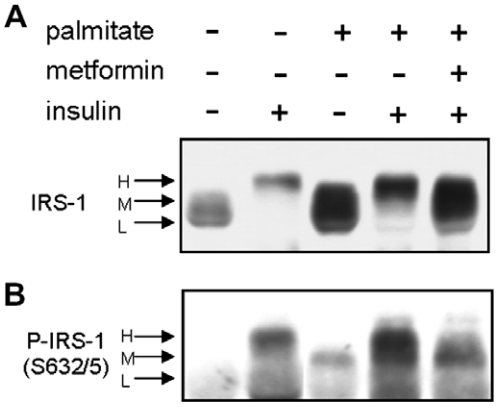

Fig. 3. Effects of insulin on electrophoretic mobility and phosphorylation state of IRS-1 in hepatocytes. The protocol for hepatocyte culture, exposure to palmitate and treatment with metformin was as in Fig. 1. Insulin stimulation (3.12 nM) was for 1 h before cell harvest. (A) Image of an immunoblot using antibodies to total IRS-1. (B) Image of an immunoblot with antibodies reacting only with IRS-1 phosphorylated at S632/S635. The experiment was repeated three times with similar results.

In immunoblots with phosphospecific antibodies recognizing IRS-1 only when phosphorylated at S632/S635, there was virtually no signal in control hepatocytes in the basal state (Fig. 3, bottom panel). Following stimulation of control cells with insulin, IRS-1 phosphorylated at S632/S635 became discernible as a band corresponding in migration to position *H*. In palmitate hepatocytes, the presence of phosphorylated IRS-1 was detectable already in the basal state as a faint band at position *M*. The degree of phosphorylation in the palmitate hepatocytes was augmented by insulin, and this was accompanied by an electrophoretic mobility shift from position *M* to *H*. Importantly, pretreatment of the palmitate cells with metformin inhibited the insulin stimulation of S632/S635 phosphorylation and reduced the mobility shift of IRS-1.

Activation of AMPK after metformin treatment of hepatocytes in culture with palmitate

The mTOR/S6K1 signaling module is under negative control by AMPK [19]. An explanation for the hyperactivity of the mTOR/S6K1 cascade in hepatocytes incubated with palmitate might be a decrease in the AMPK tone as a result of excess ATP production through fatty acid oxidation. To test this hypothesis, we measured the phosphorylation of residue T172 in the α subunit of AMPK. Phosphorylation at this site by an upstream kinase is known to correlate with AMPK activity [20]. Additionally, the phosphorylation of a key substrate of AMPK, the regulatory lipogenic enzyme acetyl-CoA carboxylase (ACC), was examined as an index of cellular AMPK activity. Immunoblotting using phosphospecific antibodies showed that the ACC phosphorylation status at the S79 residue targeted by AMPK was no different in hepatocytes in control medium and in palmitate enriched medium. By contrast, hepatocytes cultured with palmitate and treated with metformin displayed clearcut hyperphosphorylation of S79 in ACC (Fig 4A). These changes were paralleled by changes in the phosphorylation of AMPK at T172 in the three group of hepatocytes. The level of T172 phosphorylation in the α subunit of AMPK was essentially unchanged during culture with palmitate, and metformin caused an approximately 5-fold elevation of this level (Fig 4B and C).

Discussion

Culture of primary rat hepatocytes in medium supplemented with palmitate was sufficient to induce insulin resistance. This effect was manifested by a rightward shift in the dose–response curve for insulin stimulation of PKB phosphorylation and activity. By contrast with the inhibition of insulin-dependent PKB activation, the mTOR/S6K1 kinase cascade downstream of PKB appeared to be overstimulated in palmitate-treated hepatocytes. Metformin, apparently acting via stimulation of AMPK, was able to reduce the activity of the mTOR/S6K1 pathway and to re-sensitize insulin-dependent PKB signaling in palmitate hepatocytes.

Mice with targeted disruption of the S6K1 gene were shown to retain better whole body insulin sensitivity during high fat feeding compared to their wild type counterparts, suggesting a role of S6K1 in the pathogenesis of lipid-induced insulin resistance [22]. Supporting this suggestion, a study by Marette and collaborators showed that the mTOR/S6K1 cascade was overstimulated in the livers of rats fed a high fat diet for 4 weeks, at a time when insulin signaling at the level of PKB was depressed [23]. Here, similar alterations in cell signaling were induced in isolated hepatocytes after relatively short term culture in the pres-

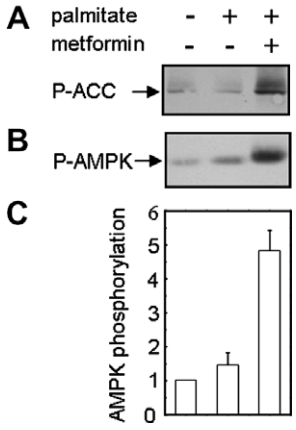

Fig. 4. Effects of palmitate and metformin on phosphorylation status of ACC and AMPK. The hepatocyte culture protocol was as in Fig. 1. (A) Image of an immunoblot of hepatocyte protein reacted with antibodies to ACC phosphorylated at S79. (B) Immunoblot of protein samples from the same hepatocytes reacted with antibodies to AMPKα phosphorylated at T172. (C) Plot from scanning data of phosphorylated AMPKα from three separate hepatocyte experiments given as means ± SEM. Values expressed as—fold increases compared to control hepatocytes.

ence of palmitate, at concentrations corresponding to the high physiological range for total fatty acids in plasma.

The mTOR kinase is under positive control by both the insulin-activated PI3K/PKB pathway and incompletely characterized nutrient sensing systems affected primarily by fluctuations in intracellular amino acid levels. Although mTOR is well-known to be activated under conditions of nutrient abundance, very few investigations addressed a putative link between supply of fatty acids to cells and mTOR activity. A study by Jefferson and colleagues [24] showed that mTOR activity was reduced in the hearts of rats that were treated with niacin to lower the plasma level of free fatty acids. Here, the mTORC1/S6K1 signaling system was overstimulated in hepatocytes cultured with palmitate. The mechanism responsible for this activation remains unknown. Recently, AMPK was shown to inhibit mTOR activity through functional inhibition of the small GTP binding protein termed Ras homolog enhanced in brain (Rheb), itself an activator of mTOR [19]. We hypothesized that delivery of palmitate to hepatocytes in culture might provide a substrate for mitochondrial β oxidation and lead to increased ATP production, in turn causing a drop in ambient AMPK activity and a rise in mTOR activity. However, our results showed that AMPK phosphorylation and activity levels were not reduced in hepatocytes after 22 h of culture with palmitate. In future studies, it will be interesting to investigate possible links between fatty acid availability or metabolism and novel mechanisms of regulation of mTOR mediated by the so-called proline rich Akt substrate 40 (PRAS40) [25], the MAP4K3 protein kinase [26] or the protein known as regulated in development and damage responses 1 (REDD1) [27].

Metformin is a classical activator of AMPK, although the molecular mechanism of activation by the drug is still not completely elucidated [20]. As expected from earlier work [21], metformin activated AMPK in the present hepatocytes. More interestingly, in the hepatocytes cultured with palmitate, metformin reduced the mTOR and S6K1 activity levels toward control values. In particular, the disproportionate ratio of mTOR activation on PKB activation prevailing in these cells after insulin stimulation was dramatically lowered by metformin. In tumor cells engineered to overexpress signaling proteins, the pathway by which AMPK negatively controls mTOR activity involves AMPK-mediated phosphorylation of TSC2 and stimulation of the GTPase activating function of this protein toward Rheb, a small G protein activator of mTOR [28]. From the present results, it would appear that this pathway might also be operative in the physiologically and metabolically relevant sytem represented by primary hepatocytes.

The elevated activity of the mTOR/S6K1 cascade in hepatocytes cultured with palmitate was accompanied by hyperphosphorylation of IRS-1 at S632/S635 compared to control hepatocytes, both in the basal state and following insulin stimulation. Phosphorylation of IRS-1 on multiple serine residues can perturb insulin signaling by several mechanisms, including decreased steady-state level of IRS-1 due to accelerated IRS-1 proteasomal degradation, or impaired interaction of IRS-1 with the insulin receptor or the p85 regulatory subunit of PI3K [29]. In IRS-1, serine residues in the 632-SPKS-635 motif of the rodent sequence are known targets for phosphorylation by mTORC1. Phosphorylation at these sites was shown to result in decreased insulin-stimulated association of IRS-1 with the p85 subunit of PI3K [17]. Therefore, overstimulation of mTOR/S6K1 and hyperphosphorylation of S632/S635 in IRS-1 may provide a potential mechanism which could account, at least in part, for the depression of PI3K/PKB-mediated insulin signaling in primary cultures of hepatocytes in presence of palmitate. Furthermore, the attenuation of the mTOR/S6K1 system and the decrease in IRS-1 phosphorylation at S632/S635 after metformin treatment might be a critical mechanism underlying the re-sensitization of these hepatocytes to insulin.

Acknowledgments

We thank Asllan Gjinovci for assistance in the isolation of hepatocytes and Françoise Assimacopoulos-Jeannet for advice. Research supported by Grant 310000–111797 from Swiss NSF.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.004.

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