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Phosphorylation and inactivation of HMG-CoA reductase at the AMP-activated protein kinase site in response to fructose treatment of isolated rat hepatocytes

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We have previously shown that incubation of isolated hepatocytes with fructose leads to elevation of AMP and activation of the AMP-activated protein kinase. We now show that this treatment causes marked inactivation of HMG-CoA reductase. Using immunoprecipitation from the microsomal fraction of ^{32}P-labelled cells, we also show that this treatment leads to a 2.6-fold increase in the phosphorylation of the 100 kDa subunit of HMG-CoA reductase. Successive digestion of this ^{32}P-labelled subunit with cyanogen bromide and endoproteinase Lys-C confirmed that Ser-871, the site phosphorylated in cell-free assays by the AMP-activated protein kinase, was the only site phosphorylated under these conditions.

HMG-CoA reductase; Cholesterol synthesis; Protein kinase; Phosphorylation site; Protein phosphorylation; Hepatocyte

1. INTRODUCTION

The principal regulatory step in the biosynthetic pathway of cholesterol and other isoprenoid compounds, the conversion of HMG-CoA to mevalonate, is catalysed by HMG-CoA reductase. This enzyme is subject to complex multivalent control mechanisms [1], including acute regulation of the activity of the enzyme by reversible phosphorylation [2]. Several protein kinases phosphorylate and inactivate HMG-CoA reductase in cell-free assays, including the AMP-activated protein kinase (formerly termed HMG-CoA reductase kinase) [3,4], protein kinase C [5] and a calmodulin-activated protein kinase [6]. The AMP-activated protein kinase is the major Ca^{2+}-independent HMG-CoA reductase kinase in rat liver [4] and appears to have a general role in the regulation of lipid metabolism [2]. This kinase is activated allosterically by AMP, which also promotes its further activation due to phosphorylation by a distinct kinase [7]. Recently we have demonstrated that the AMP-activated protein kinase [8], protein kinase C and calmodulin-dependent multiprotein kinase [9] all phosphorylate a single identical site on HMG-CoA reductase, corresponding to Ser-871 in the hamster enzyme (Ser-872 in the human sequence). This site is phosphorylated in intact rat liver under conditions where HMG-CoA reductase is largely in the inactive form [8]. We now show that this site also becomes phosphorylated when AMP is elevated in intact rat hepatocytes, and that this is the only site phosphorylated on the 100 kDa subunit under these conditions. Increased phosphorylation at this site is associated with a marked inactivation of HMG-CoA reductase.

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2. MATERIALS AND METHODS

2.1. *Materials*

[^{14}C]HMG-CoA and [^{32}P]phosphate were from Amersham International, and [^{3}H]mevalonolactone from New England Nuclear. Leupeptin was from The Peptide Institute, Japan. Tween-20 was from Bio-Rad. Nitrocellulose was from Schleicher & Schuell. HEPES, mannitol, NADP, glucose-6-phosphate dehydrogenase, sodium dodecyl sulfate, deoxycholic acid (Na salt), Triton X-100, and iodoacetamide were from Sigma, Dorset, UK. Digitonin (high purity) from Calbiochem was further purified as described [10]. Sources of other materials have been previously described [4,7,8].

2.2. *Isolation and incubation of cells*

Before hepatocyte isolation, male rats were fed for 6 days on standard laboratory chow supplemented with 2% (by mass) cholestyramine. For the last 2 days, the diet was also supplemented with 0.2% (by mass) symvastatin. Hepatocytes were isolated and incubated at 100 mg wet wt./ml as previously described [7]. Cells were pre-incubated 90 minutes before addition of fructose (2 M, in incubation medium). For ^{32}P-labeling (0.25 mCi/ml), the phosphate concentration in the medium was reduced to 0.2 mM.

2.3. *Immunoprecipitation of ^{32}P-labelled HMG CoA reductase*

To minimize phosphorylation of HMG-CoA reductase during harvesting of cells, 16 ml aliquots of cell suspension were diluted with 35 ml of ice-cold Krebs–Henseleit buffer before centrifugation [11]. They were resuspended in 4 ml of ice-cold hypotonic homogenisation buffer (50 mM Tris/HCl, pH 7.2, at 4°C, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 50 mM NaF, 1 mM Na pyrophosphate, with the following proteinase inhibitors: 1 mM phenylmethane sulphonyl fluoride, 0.1 mM leupeptin, 0.1 mM TPCK, 0.1 mM TLCK, 0.1 mM benzamid-

ine, and 0.1 mM soybean trypsin inhibitor), and homogenised with 40 strokes of a ground-glass homogeniser. 1 ml of homogenisation buffer containing 1.25 M sucrose was added, the homogenate centrifuged (16,000×g, 15 min, 4°C), and the supernatant recentrifuged (100,000×g, 4°C). The microsomal pellet was resuspended in 1 ml of immunoprecipitation buffer (1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) sodium dodecyl sulphate, 20 mM HEPES, pH 7.2, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, plus proteinase inhibitors as before) and 10 μl of rabbit anti-HMG CoA reductase serum added. After incubation on ice for 60 min, 40 μl of protein A–agarose was added, and incubation continued for 60 min with gentle orbital shaking. Immune complexes were recovered by centrifugation (12,000×g, 10 s, 4°C). Immunoprecipitates were analysed on 5–15% polyacrylamide gradient gels as described previously [8].

2.4. *Phosphopeptide analysis of ^{32}P-labelled HMG-CoA reductase*

The purified 53 kDa fragment of rat liver HMG CoA reductase was phosphorylated using [γ-^{32}P]ATP and purified AMP-activated protein kinase as previously described [8]. HMG-CoA reductase was also immunoprecipitated from ^{32}P-labelled cells, purified by SDS-PAGE, and transferred to nitrocellulose using a Bio-Rad Transblot apparatus. The 100 kDa subunit was located on a Molecular Dynamics Phosphorimager, excised, and eluted from the nitrocellulose using 70% formic acid. The ^{32}P-labelled 53 kDa fragment or 100 kDa subunit were then subjected to successive digestion wth CNBr and endoproteinase Lys-C, and analysed by thin-layer isoelectric focussing [8]. Labelled peptides were detected on a Molecular Dynamics Phosphorimager.

2.5. *Other procedures*

AMP-activated protein kinase was partially purified from hepatocyte suspensions by polyethylene glycol precipitation and assayed as previously described [7]. HMG-CoA reductase was assayed (4 min, 37°C) in situ in digitonin-permeabilised hepatocytes [12] except that we used 50 μg digitonin per mg cell protein, 472.5 mU glucose-6-phosphate dehydrogenase, and 10,000 dpm/nmol [^{14}C]HMG-CoA. Immunodetection of immunoprecipitated HMG-CoA reductase that had been electroblotted to nitrocellulose was carried out as follows, with all reagents being diluted in TBS-Tween (25 mM Tris/HCl, pH 7.5, at 20°C, 150 NaCl, 0.05% (v/v) Tween-20). The blot was incubated successively for 1 h each in 3% (w/v) low-fat milk powder, sheep anti-HMG-CoA reductase IgG (50 μg/ml) plus 1% (w/v) milk powder, 0.25% (v/v) biotinylated donkey anti-sheep IgG plus 1% (w/v) milk powder, and 0.25% (by vol.) streptavidin-biotinylated horseradish peroxidase complex plus 1% (w/v) milk powder. Between each incubation the blot was washed five times for 5 min with TBS-Tween. HMG-CoA reductase was visualised using the ECL detection kit (Amersham). Protein concentrations were measured by the dye-binding method of Bradford [13].

3. RESULTS

3.1. *Effect of fructose on HMG-CoA reductase activity*

As reported previously [7], treatment of isolated rat hepatocytes with 20 mM fructose caused a transient activation of the AMP-activated protein kinase, reaching a peak at 5–15 min and then slowly reverting to the control activity (Fig. 1B). We assayed HMG-CoA reductase in the same cells, using a method in which the enzyme is measured in situ in digitonin-permeabilized cells [12]. As expected, fructose treatment caused a reciprocal change in HMG-CoA reductase activity. The activity was dramatically reduced by 5 min, remained low up to 20 min, and then reverted to a value at or above the control (Fig. 1).

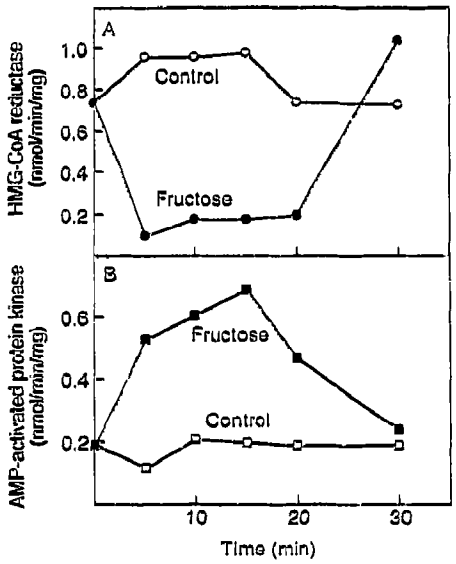

Fig. 1. (A)HMG-CoA reductase activity and (B) AMP-activated protein kinase activity in hepatocyte extracts made at various times after addition of fructose to the cells. Similar results were obtained in three other experiments.

3.2. *Effect of fructose on phosphorylation of HMG-CoA reductase*

Hepatocytes were labelled for 90 min with [^{32}P]phosphate before treatment with or without fructose for 10 min. HMG-CoA reductase was purified by immunoprecipitation using rabbit antibodies raised against the 53 kDa fragment of the enzyme, followed by SDS-PAGE and transfer to nitrocellulose. Fig. 2 (panel A) shows that fructose caused a marked stimulation of ^{32}P-labelling of the 100 kDa polypeptide of HMG-CoA reductase. In six separate experiments the increase in ^{32}P-labelling of the 100 kDa subunit was 2.6 ± 0.6-fold (mean ± S.E.M., $P<0.05$). The polypeptide of 120 kDa, whose ^{32}P-labelling was also increased by fructose, appeared to be unrelated to HMG-CoA reductase because it was also precipitated with control serum (not shown) and was not consistently observed. In some experiments (e.g. panel B) the antibody also precipitated polypeptides which migrated in the range 50–60 kDa, and for which ^{32}P-labelling was also increased after fructose treatment. The proteolytic fragments of HMG-CoA reductase are known to migrate in this region [14], and variable amounts of these polypeptides were observed, despite the use of a battery of proteinase inhibitors. Since we needed to be sure that the increased labelling of the 100 kDa subunit was not merely due to different degrees of proteolysis, we also probed the blots using sheep antibodies distinct from those used for immunoprecipitation. Panel C shows that both the 100 and 60 kDa polypeptides were recognized by this anti-

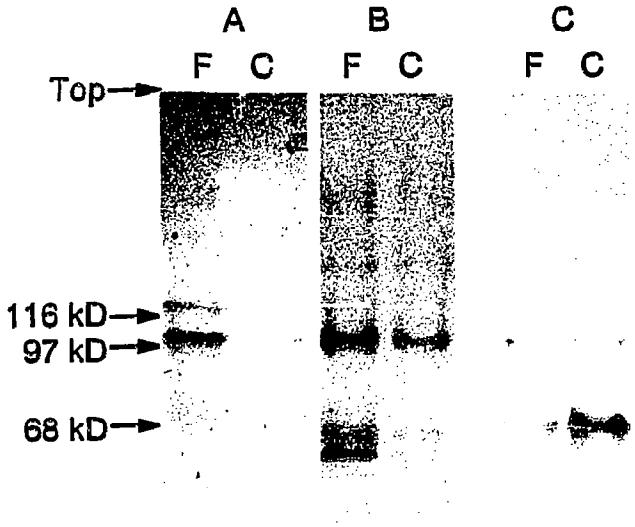

Fig. 2. (A,B) Autoradiograms and (C) immunoblot of immunoprecipitates from ^{32}P-labelled cells which had been treated with (F) or without (C) fructose. Anti-HMG-CoA reductase immunoprecipitates made using rabbit antibodies were analysed by SDS-PAGE and transferred to nitrocellulose before detection using autoradiography (A,B) or sheep anti-HMG-CoA reductase antibodies (C). Panels A and B are separate experiments; panels B and C are the same nitrocellulose blot.

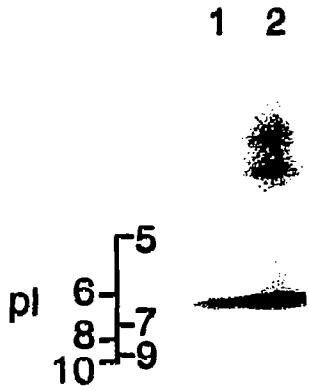

Fig. 3. Thin-layer isoelectric focussing of a CNbr/endoproteinase Lys-C digest of ^{32}P-labelled HMG-CoA reductase. (Lane 1) 100 kDa subunit from fructose-treated (10 min) ^{32}P-labelled hepatocytes; (lane 2) the 53 kDa catalytic fragment phosphorylated using [γ-^{32}P]ATP and purified AMP-activated protein kinase. Labeled peptides were detected on a Molecular Dynamics Phosphorimager. The isoelectric point (pI) scale, which is a rough guide only, was drawn by comparison with the mobility of coloured marker proteins.

body, but that the recovery of the 100 kDa subunit was identical in the two samples. There was some evidence that further degradation of the 60 kDa fragments to smaller fragments not recognized by the sheep antibody was increased in the samples from fructose-treated cells.

3.3. *Identification of the site phosphorylated in response to fructose*

The area of nitrocellulose containing the ^{32}P-labelled 100 kDa subunit from an experiment similar to that shown in Fig. 2A was excised, and the polypeptide eluted with formic acid. The polypeptide was then subjected to successive digestions with cyanogen bromide and endoproteinase Lys-C. As a marker, a sample of the purified 53 kDa fragment of rat liver HMG-CoA reductase was phosphorylated using [γ-^{32}P]ATP and purified AMP-activated protein kinase and analysed in the same way. We have shown previously that this procedure generates from the 53 kDa fragment a single ^{32}P-labelled peptide with the sequence, VHNRSK, containing the serine residue equivalent to Ser-871 on the hamster enzyme [8]. The two samples were run side-by-side on thin layer isoelectric focussing. Fig. 3 (lane 2) shows that the sample of 53 kDa fragment phosphorylated by the AMP-activated protein kinase generated a major phosphopeptide with the same pI (6.5) as the VHNRSK peptide analysed previously [8], plus two minor acidic phosphopeptides. The 100 kDa subunit from ^{32}P-labelled, fructose-treated cells yielded only a single phosphopeptide (Fig. 3, lane 1), which exactly co-migrated with the pI 6.5 phosphopeptide in lane 2.

4. DISCUSSION

To our knowledge this paper represents for the first time that phosphorylation of the 100 kDa subunit of HMG- CoA reductase has been demonstrated directly by ^{32}P- labelling in intact cells, although Beg et al. [6] reported ^{32}P labelling of the 53 kDa fragment in rats injected with [^{32}P]phosphate. Our results provide strong evidence that the AMP-activated protein kinase is a physiological regulator of HMG-CoA reductase. Incubation of rat hepatocytes with 20 mM fructose causes a large fall in ATP and concomitant rise in AMP level in the cell [7]. This is thought to be due to the trapping of phosphate as esters of fructose and trioses, which inhibits resynthesis of ATP from ADP, and leads to conversion of ADP to AMP via adenylate kinase [16]. These effects are transient, and normal nucleotide levels are regained once the fructose is metabolized. Correlating with the rise in AMP there is a dramatic but transient activation of the the AMP-activated protein kinase, due to increased phosphorylation as well as allosteric activation [7]. In this paper we show that this causes a marked reciprocal inactivation of HMG-CoA reductase (Fig. 1). Reactivation of HMG-CoA reductase does not occur until the kinase activity has returned almost to the basal level (e.g. at 30 min in Fig. 1). This is similar to results obtained previously with acetyl-CoA carboxylase [7], and suggests that only a small activation of the kinase is required in order to obtain complete conversion of acetyl-CoA carboxylase or HMG-CoA reductase to their inactive, phosphorylated forms.

Fig. 2 shows that incubation with fructose for 10 min also caused a marked stimulation of phosphorylation of the 100 kDa subunit of HMG-CoA reductase, although the results of some experiments were complicated by degradation to the 50–60 kDa catalytic fragments. Despite the inclusion of a battery of proteinase inhibitors in the homogenization medium, the proteolysis most likely occurred after homogenization rather than in the intact cells, because it was not observed in all experiments (e.g. Fig. 2A).

The results in Figure 3 show that the AMP-activated protein kinase site is the only site phosphorylated in the 100 kDa subunit under these conditions. On isoelectric focussing a single ^{32}P-peptide was obtained which exactly co-migrated with the VNHRSK peptide obtained by phosphorylation of the 53 kDa fragment of HMG-CoA reductase using purified AMP-activated protein kinase. This peptide contains a single phosphorylatable residue, equivalent to Ser-871 in the hamster enzyme and Ser-872 in the human enzyme (the complete sequence of the rat enzyme is not yet known) [8]. We have previously shown that this residue is phosphorylated in intact rat liver sampled under conditions (absence of cold- or freeze-clamping) which cause elevation of AMP [8]. However, the non-radioactive methods used in that analysis did not allow us to determine whether any other sites were phosphorylated. We can now be confident that Ser-871/872 is the only major site phosphorylated on HMG-CoA reductase in rat hepatocytes under these conditions.

Ser-871/872 is also phosphorylated in cell-free assays by protein kinase C and calmodulin-dependent multiprotein kinase [9]. However, while the AMP-activated protein kinase is dramatically activated in response to fructose, there are no grounds for believing that the other two protein kinases would be. In addition, others have shown [17] that phorbol esters, which activate protein kinase C, do not cause inactivation of HMG-CoA reductase in rat hepatocytes. Elevation of Ca^{2+} using ionophores or combinations of vasopressin plus glucagon do lead to inactivation of HMG-CoA reductase, but this is not blocked by calmodulin antagonists [17], making it unlikely that the effect is due to phosphorylation by a calmodulin-dependent protein kinase. In fact Ca^{2+} ionophores caused elevation of AMP [17] and so the effect in that case may have been brought about by activation of the AMP-activated protein kinase.

Taken together with previous results demonstrating inactivation of acetyl-CoA carboxylase in response to fructose treatment [7], these findings suggest that one function of the AMP-activated protein kinase is as a type of stress response. Any treatment of the cells which causes a depletion of ATP, and consequent rise in AMP, would cause activation of the kinase and would tend to switch off both fatty acid and cholesterol synthesis. These two pathways are very active in rat liver, and represent major consumers of ATP and NADPH.

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