INTRA- AND INTERCELLULAR MECHANISMS REGULATING GLUCOSE METABOLISM IN THE LIVER

INTRA– EN INTERCELLULAIRE MECHANISMEN BETROKKEN BIJ DE REGULATIE VAN HET GLUCOSE METABOLISME IN DE LEVER

PROEFSCHRIFT

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SAMENVATTING

In dit proefschrift worden de intra- en intercellulaire mechanismen die een rol spelen bij de regulatie van het glucose metabolisme in de lever beschreven.

Fructose-1,6-bisphosphatase, een enzym betrokken bij de novo synthese van glucose, werd geactiveerd door het hormoon glucagon. In geïsoleerde parenchymale lever cellen verhoogde glucagon de Vmax van fructose-1,6-bisphosphatase. Deze verhoging van de Vmax kon teniet worden gedaan door middel van gelfiltratie van het enzym, hetgeen aantoont dat activatie van fructose-1,6-bisphosphatase door glucagon geschiedt via een activator van het enzym.

In humane lever werd de eiwitphosphorylering bestudeerd; de verkregen resultaten werden vergeleken met proefdierstudies. In de cytosol fractie van humane lever werden drie eiwitten gephosphoryleerd door cAMP-afhankelijke proteine kinase(n), twee eiwitten door Ca^{2+}-afhankelijke proteine kinase(n) en vijf eiwitten werden gephosphoryleerd door beide types proteine kinase. De cAMP-afhankelijke phosphorylering van L-type pyruvaat kinase en de cAMP- en Ca^{2+}-onafhankelijke phosphorylering van een eiwit met een molecuulgewicht van 68.000 werd geremd door gephosphoryleerde hexoses. Geconcludeerd wordt dat de eiwit phosphorylering in humane lever op vergelijkbare wijze verloopt als in rattelever.

De belangrijkste metabole routes betrokken bij het handhaven van de glucose homeostase in het bloed, zijn gelocaliseerd in de parenchymale levercellen. Behalve de parenchymale cellen bevat de lever ook Kupffer cellen, endotheelcellen, "fat storing" cellen en pitcellen. In de literatuur is gesuggereerd dat niet-parenchymale levercellen wellicht een rol spelen bij de transductie van sommige glycogenolytische stimuli zoals de tumor promotor PMA. In

rattelever werd de rol van de niet-parenchymale levercellen in de regulatie van de eiwitphosphorylering en glycogenolyse in parenchymale levercellen bestudeerd. De phosphoryleringsgraad van glycogeen phosphorylase en van een eiwit met een molecuulgewicht van 47,000 werd verhoogd door geconditioneerde media van Kupffer en leverendotheelcellen. De phosphoryleringsgraad van een gephosphoryleerd secretie-eiwit met een molecuulgewicht van 63.000 werd verlaagd. Dezelfde effecten konden teweeggebracht worden door prostaglandine E_1, E_2 en D_2. Geconcludeerd kan worden dat prostaglandines, zoals gevormd door endotheel en Kupffer cellen, de eiwitphosphorylering in parenchym cellen kunnen beïnvloeden.

In geperfundeerde lever werd de glycogenolyse gestimuleerd door de tumor promotor PMA. In geïsoleerde parenchymale levercellen kon PMA echter de glycogenolyse niet stimuleren. De hypothese dat niet-parenchymale levercellen een rol spelen bij de stimulatie van glycogenolyse door PMA werd bevestigd doordat gevonden werd dat glycogenolyse in parenchymale levercellen gestimuleerd kon worden door geconditioneerde media van Kupffer en leverendotheelcellen. De productie van de belangrijkste prostaglandine in de lever, prostaglandine D_2, werd gestimuleerd door PMA. Prostaglandine D_2 was in staat zowel in de geperfundeerde lever als in geïsoleerde parenchymale levercellen de glycogenolyse te stimuleren. Geconcludeerd kan worden dat PMA de glycogenolyse in geperfundeerde lever kan stimuleren via een primaire interactie met niet-parenchymale levercellen waarna prostaglandine D_2 vervolgens de parenchymale cellen activeert.

Endotoxine, een bacterieel toxine, kan de glucose homeostase verstoren doordat het glycogeen metabolisme en de gluconeogenese in de lever worden beïnvloed. Omdat endotoxine uit de circulatie wordt verwijderd door Kupffer cellen bestaat de mogelijkheid dat

endotoxine het glucose metabolisme in de parenchymale levercellen beïnvloedt met behulp van Kupffer cellen.

In geperfundeerde rattelever stimuleerde endotoxine de glycogenolyse maar in geïsoleerde parenchymale cellen trad geen effect op. De stimulatie van de glycogenolyse in de geperfundeerde lever kon worden geblokkeerd door aspirine. Endotoxine stimuleerde de productie van prostaglandine D_2 in de lever, met een tijdsafhankelijkheid die een intermediaire rol in de stimulatie van de glycogenolyse toestaat.

Er wordt geconcludeerd dat endotoxine de glycogenolyse in de lever via eenzelfde intercellulair mechanisme stimuleert als PMA, waarbij prostaglandine D_2 als intercellulaire boodschapper dient.

De intercellulaire communicatie, zoals gedefinieerd in dit proefschrift, voegt een nieuwe dimensie toe aan de complexe regulatie van de glucose homeostase door de lever en is waarschijnlijk van belang onder pathophysiologische condities.

SUMMARY

The regulation of glucose metabolism in the liver by intra- and intercellular mechanisms was studied.

Fructose-1,6-bisphosphatase, an enzyme involved in de novo synthesis of glucose was found to be stimulated by glucagon in isolated parenchymal liver cells. Glucagon increased the Vmax of fructose-1,6-bisphosphatase. This increase could be abolished by gel-filtration of the enzyme, indicating that stimulation of fructose-1,6-bisphosphatase is caused by an activator of the enzyme.

In human liver, protein phosphorylation was studied in order to extend results from animal studies to the human situation. In the human liver cytosolic fraction three proteins were phosphorylated by cAMP-dependent protein kinase, two proteins by Ca^{2+}-dependent protein kinase(s) and five proteins were phosphorylated by both types of protein kinases. The cAMP-dependent phosphorylation of L-type pyruvate kinase and the cAMP-and Ca^{2+}-independent phosphorylation of a protein with a molecular weight of 68,000 was inhibited by phosphorylated hexoses. Protein phosphorylation in human liver was found to be similar to that in rat liver.

The major metabolic pathways involved in the maintenance of glucose homeostasis in the blood, are located in the parenchymal liver cells. In addition to parenchymal cells the liver contains Kupffer cells, endothelial cells, fat storing cells and pit cells. In the literature it has been suggested that non-parenchymal liver cells might play a role in the transduction of certain glycogenolytic stimuli, such as the tumor promotor PMA. In rat liver the role of non-parenchymal liver cells in the intercellular regulation of protein phosphorylation and glycogenolysis in

parenchymal liver cells was studied. The phosphorylation state of glycogen phosphorylase and of a protein with a molecular weight of 47,000 was increased by conditioned media of Kupffer and endothelial liver cells. The phosphorylation state of a secretory phosphoprotein with a molecular weight of 63,000 was decreased. The same effects could be obtained with prostaglandins E_1, E_2 and D_2. So, Kupffer and endothelial liver cells can influence protein phosphorylation in parenchymal cells.

Glycogenolysis in perfused liver was stimulated by the tumor promotor PMA. In isolated parenchymal liver cells glycogenolysis was not stimulated by this agent. The suggestion that non-parenchymal liver cells mediate the stimulation by PMA was supported by the activation of glycogenolysis in parenchymal cells by conditioned media of Kupffer and endothelial liver cells. Prostaglandins were shown to be the active factor(s) in these media and production of the most prominent prostaglandin produced by Kupffer and endothelial liver cells, prostaglandin D_2, was shown to be increased by PMA added to perfused liver. Furthermore, prostaglandin D_2 also activated glycogenolysis in isolated parenchymal liver cells. It is concluded that phorbol esters stimulate glycogenolysis in perfused liver via a primary interaction with non-parenchymal cells, leading to release of prostaglandin D_2 wich then acts on parenchymal cells.

Endotoxin, a bacterial toxin, can elicit drastic changes in glucose homeostasis and has been reported to affect glycogen metabolism and gluconeogenesis in the liver. Since endotoxin is removed from the circulation by Kupffer cells, the possiblity that these cells modulate glucose metabolism in parenchymal liver cells in response to endotoxin was studied. In the perfused rat liver endotoxin stimulates glycogenolysis but endotoxin has no

effect on glycogenolysis in isolated parenchymal cells. The stimulation of glycogenolysis in the perfused liver could be blocked by acetylsalicylic acid. Furthermore endotoxin stimulated the production of prostaglandin D_2 by the perfused liver, with a time course which is compatible with an intermediary role in the stimulation of glycogenolysis.

It is concluded that endotoxin stimulates glycogenolysis in the liver via a similar intercellular mechanism to PMA, involving prostaglandin D_2 as an intercellular mediator.

This demonstration of intercellular communication adds a new dimension to the complex regulation of glucose homeostasis by the liver. This process is likely to operate under pathological conditions.

1. INTRODUCTION

1.1. Liver morphology

One of the major functions of the liver is the uptake from the circulation of substrates derived from the intestine and their subsequent metabolism, storage and redistribution to blood and bile. The morphology of the liver is designed to fulfil these functions, which require an adequate exchange between cells and the blood. The presence of different cell types in the liver enables the further specification and modulation of this process.

The predominant cell type in the liver is the parenchymal cell. Parenchymal cells, which have eight or more sides, are arranged in plates, which are usually one cell thick (Fig. 1A). The parenchymal cell plates are interconnected, forming a continuous three dimensional network. Blood from the portal vein and liver arteries is transported to the central vein along the plates, through the sinusoids, which lie between the parenchymal cell plates. Cell membranes of adjacent parenchymal cells form the bile caniculi. Parenchymal cells are the largest cells (13-30 μm) present in the liver and represent about 60% of the cells, accounting for about 80% of the liver volume. Major metabolic functions of the liver including the regulation of the body's energy balance, e.g. storage and breakdown of glycogen and *de novo* synthesis of glucose, take place in the parenchymal cells.

Besides parenchymal cells, the liver contains Kupffer cells, endothelial liver cells, fat storing cells and pit cells (2). About 10% of the cells in the liver are Kupffer cells. Kupffer cells are usually stellate in shape and are preferentially distributed in the sinusoids around the portal tract. The stellate extensions of the Kupffer cells are attached to endothelial

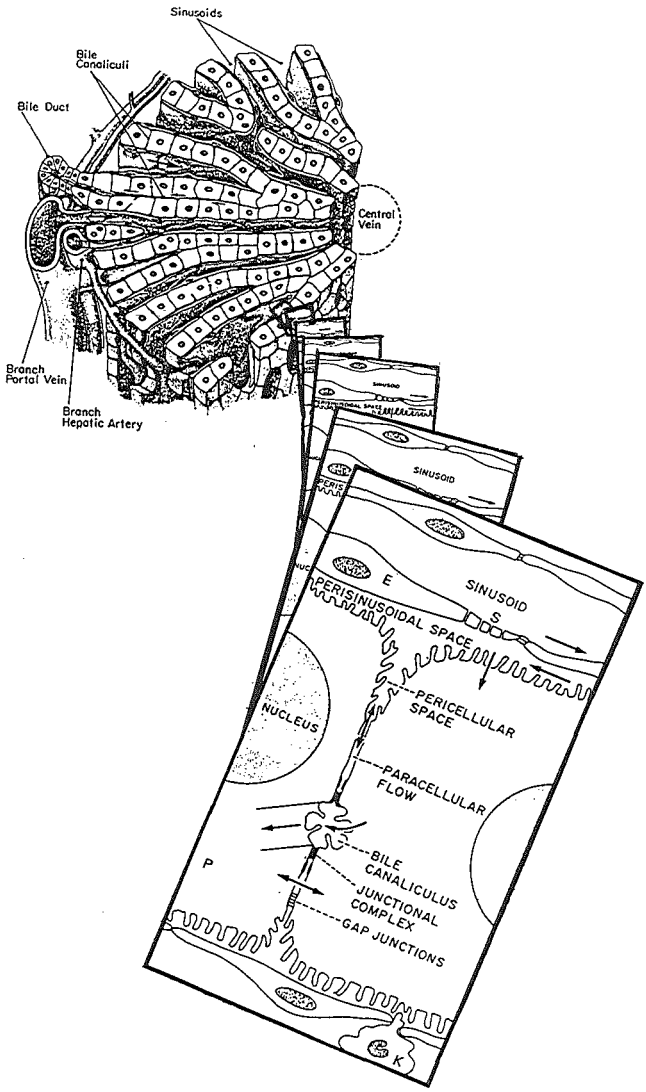

Figure 1.Schematic representation of part of a liver lobule and a cross-section of a liver cell plate showing different liver cell types in relation to the perihepatocellular spaces.
E: endothelial cell; K: Kupffer cell; P: parenchymal cell; S: sieve plate. (adapted from ref. 1 and 7).

cells. Kupffer cells can be distinguished from other sinusoidal cells by peroxidase staining (3). Kupffer cells contain many lysosomes and pinocytotic vesicles and they constitute about 80 to 90% of the fixed macrophages of the reticuloendothelial system (4).

Endothelial liver cells represent about 25% of the cells in the liver (5). The endothelial cells form the walls of the liver sinusoids and contain typical fenestrations, which are arranged in sieve plates (Fig. 1B). Through the sieve plates, extensions of the Kupffer cells can penetrate the perisinusoidal spaces of Disse, between endothelial cells and parenchymal cells. The sieve plates provide a filtration barrier with a pore size of approximately 100 nm. amd shield the spaces of Disse from large particles in the circulating blood (6). Whereas the Kupffer cells are efficient in phagocytosis of large particles, endothelial liver cells are able to take up macromolecules from the blood mainly by selective receptor mediated endocytosis via clathrin-coated vesicles. Endothelial cells are smaller than Kupffer cells and they contain fewer lysosomes than Kupffer cells (7).

Fat storing cells, which make up only a few percent of the liver cells, typically contain fat droplets in their cytoplasm, filled with vitamin A. Fat storing cells are located in the spaces of Disse.

Finally a small number of pit cells, which have a neuroendocrine appearance, are present in the liver (8).

The specific properties of the different liver cells types enable the liver as an organ to fulfil its complex functions.

1.2 Regulation of glucose metabolism in parenchymal liver cells

The metabolic machinery for the maintenance of glucose homeostasis in the blood operates in the parenchymal liver cells. When the level of glucose in the blood is high, synthesis of glycogen is stimulated (9,10) and glycogen is stored in glycogen granules.

During fasting, glucose is initially released from glycogen stores and subsequently synthesized from pyruvate, lactate, glycerol or aminoacids. Gluconeogenesis utilizes the enzymes of glycolysis (Fig. 2) for the near-equilibrium reactions. However, at three sites different enzymes are used for glycolysis and gluconeogenesis and it is at these sites that the metabolic pathways are regulated.

Glucose metabolism in the liver is regulated by hormone action. Glycogen breakdown is regulated by hormones, mainly at the site of glycogen phosphorylase. Hormonal regulation of gluconeogenesis and glycolysis is effected at the level of the so-called substrate cycles between pyruvate and phosphoenolpyruvate, and between fructose-1,6-bisphosphate and fructose-6-phosphate (11-13). The activity of the individual enzymes in these cycles determines whether the flux is glycolytic or gluconeogenic.

Two different classes of hormones, which act via different second messengers, regulate glucose metabolism in the liver, cAMP-generating hormones (glucagon, β-adrenergic agents) and Ca^{2+}-mobilizing hormones (vasopressin, angiotensin, α-adrenergic agents). Glucagon stimulates, via an increase of cAMP, glycogenolysis and gluconeogenesis and inhibits glycolysis. Low doses of glucagon can be counteracted by insulin (14). α-Adrenergic hormones, vasopressin and angiotensin act on intracellular Ca^{2+} levels

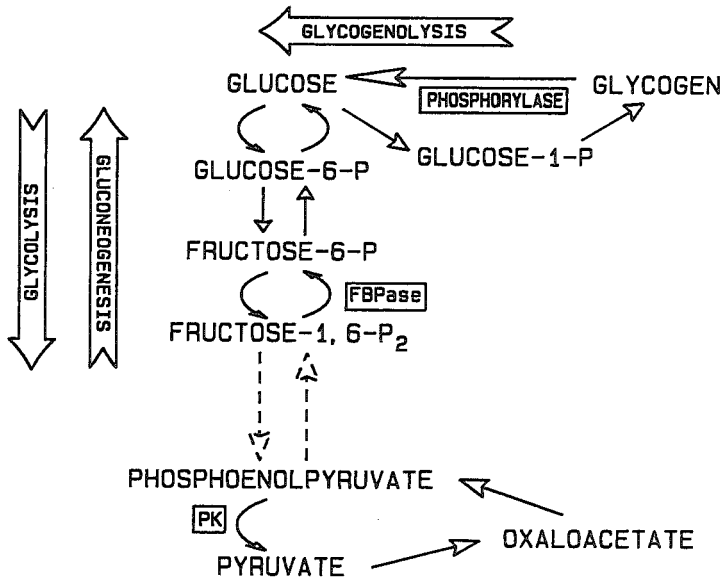

Figure 2.Glucose metabolism in the liver.
FBPase: fructose-1, 6-bisphosphatase; PK: pyruvate kinase.

and have a main effect on glycogen phosphorylase, leading to subsequent stimulation of glycogen breakdown (15).

cAMP is generated by adenylate cyclase, which is activated after a hormone (e.g. glucagon) has bound to its receptor (Fig. 3). Receptors are coupled to adenylate cyclase by guanine nucleotide regulatory proteins (16). Depending on the nature of the guanine nucleotide regulatory protein, cAMP synthesis can either be stimulated or inhibited. An increased cAMP concentration leads to the activation of cAMP-dependent protein kinase. In rat liver, cAMP-dependent protein kinase catalyzes the phosphorylation and subsequent changes in the kinetic behaviour of four enzymes involved in the control of glucose metabolism: phosphorylase, glycogen synthase, phosphofructokinase-2/fructosebisphosphatase-2 and pyruvate kinase (17). In addition to these enzymes fructosebisphosphatase-1 and phosphofructokinase-1 have also been reported to be phosphorylated; however, no clear changes in enzymatic activity have been reported for these enzymes following phosphorylation (18,19).

When phosphorylase kinase is activated by cAMP-dependent phosphorylation it catalyzes the phosphorylation of phosphorylase, which is thus activated resulting in the breakdown of glycogen (Fig. 3). Simultaneously, glycogen synthase is deactivated by cAMP-dependent phosphorylation.

cAMP-dependent protein kinase also phosphorylates phosphofructokinase-2/fructobisphosphatase-2 (20,21). Although this enzyme does not directly participate in the gluconeogenesis/glycolysis pathway, it plays an important role in the regulation of the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle (22,23). Phosphofructokinase-2/fructosebisphosphatase-2 is a bifunctional enzyme with two distinct active sites, and depen-

ding on its phosphorylation state, catalyzes the synthesis or breakdown of fructose-2,6-bisphosphate. Fructose-2,6-bisphosphate is a potent activator of phosphofructokinase-1 and an inhibitor of fructosebisphosphatase-1. Glucagon stimulates the cAMP-dependent phosphorylation of this bifunctional enzyme. Phosphorylation results in inactivation of the synthesis of fructose-2,6-bisphosphate and activation of the hydrolytic activity of the enzyme. As a result the fructose-2,6-bisphosphate concentration declines rapidly. By this mechanism glucagon can counteract activation of phosphofructokinase-1 and inhibition of fructosebisphosphatase-1, and change the flux through this cycle towards gluconeogenesis.

cAMP-dependent phosphorylation of pyruvate kinase inhibits its activity, causing the phosphoenolpyruvate concentration to rise, which favors the phosphoenolpyruvate/pyruvate cycle to operate in the gluconeogenic direction (24).

Receptor binding of the so-called Ca^{2+}-linked hormones (vasopressin, angiotensin and α-adrenergic agents) leads to the activation of phospholipase C which catalyzes the hydrolysis of polyphosphoinositides to diacylglycerol and inositol-1,4,5-triphosphate which both act as second messengers (Fig. 3) (25). Inositol-1,4,5-trisphosphate triggers the release of Ca^{2+} from endogenous stores, located in the endoplasmic reticulum (26). Diacylglycerol activates protein kinase C, which seems to play a role in the regulation of glucose metabolism in the liver by yet unknown mechanisms.

An increase in the cytosolic Ca^{2+} concentration activates Ca^{2+}-dependent protein kinases such as phosphorylase kinase. Subsequent phosphorylation of glycogen phosphorylase will lead to enhanced glycogenolysis.

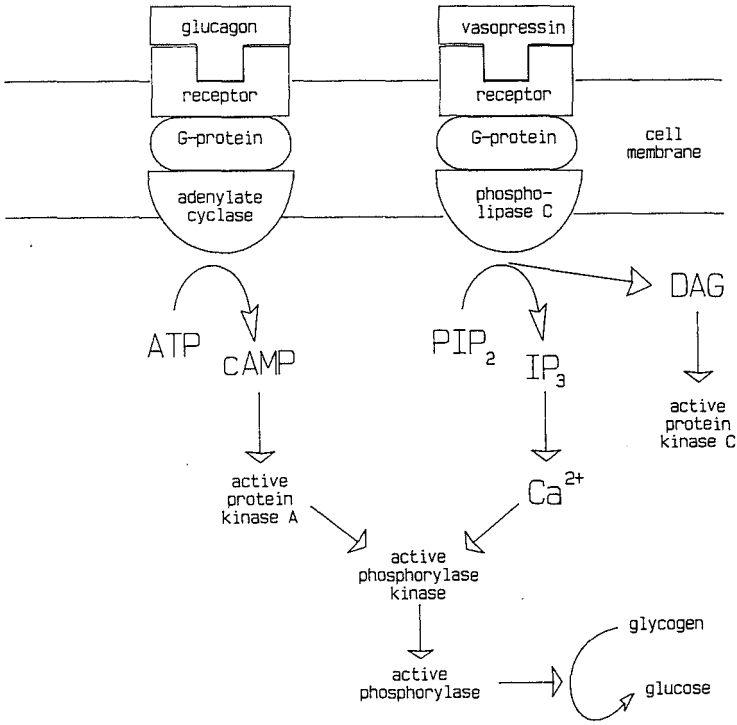

Figure 3.Mechanism of stimulation of glycogenolysis in the liver by glucagon and vasopressin.

ATP: adenosine-triphosphate; cAMP: cyclic adenosine monophosphate PIP 2: phosphatidylinositol 4, 5 bisphosphate; IP 3: inositol 1, 4, 5 trisphosphate; DAG: diacylglycerol.

Pyruvate kinase is reported (27,28) also to be phosphorylated in response to Ca^{2+}-dependent hormones (27), at the same site as it is phosphorylated by cAMP-dependent protein kinase (28,29). This phosphorylation is reported to result in a loss in enzyme activity (30) although this effect of phosphorylation has not been found by all workers (17).

In contrast to glucagon, Ca^{2+}-linked hormones (vasopressin and angiotensin) do not stimulate the phosphorylation of the bifunctional enzyme phosphofructokinase-2/fructosebisphosphatase-2 (27). Therefore the influence of hormones, such as vasopressin and angiotensin, that act strictly via phospholipase C coupled receptors, is limited to glycogen phosphorylase and possibly pyruvate kinase.

The ability of hormones to regulate the glucose metabolism in the liver, enables the liver to adapt its function to the physiological requirements of the body.

1.3. Intercellular regulation of glycogenolysis in the liver.

Glucagon and vasopressin stimulate glycogenolysis in the liver by binding to receptors located at the cell membrane of the parenchymal liver cells, resulting in the generation of second messengers, cAMP and Ca^{2+}.

Another type of regulatory mechanism has been hypothesized for the tumor promoting phorbol ester, phorbol-12-myristate-13-acetate (PMA) platelet activating factor (PAF) and heat aggregated immunoglobulin G (HAG), which involves an interaction with non-parenchymal liver cells. These agents do not directly influence glycogenolysis in isolated parenchymal cells, and it was suggested that an interaction with non-parenchymal cells induces

the production of substances which subsequently stimulate glycogenolysis in parenchymal cells.

This cellular communication hypothesis is based on the finding that PMA, PAF and HAG are able to stimulate glycogenolysis (Fig. 4A) in the perfused liver (31-35) whereas they have no effects on glycogenolysis (Fig. 4C) in isolated parenchymal cells (36-38), in contrast to glucagon (Fig. 4A+B). PAF has been reported to stimulate breakdown of phosphatidylinositol-4,5-bisphosphate in isolated parenchymal cells (34,36), so it seems unlikely that cell isolation leads to a loss of responsiveness to PAF. The ability of glucagon (35) and Ca^{2+}-mobilizing agents (36-38) to stimulate glycogenolysis in isolated parenchymal cells indicates that the intracellular regulatory mechanisms of glycogenolysis are still intact. The suggestion that non-parenchymal cells were involved in the expression of the glycogenolytic effect of PMA, PAF and HAG was based in part on the expectation that these factors would interact with non-parenchymal cells. Soluble immune complexes are known to be removed from the circulation via the Fc-receptors of the Kupffer cells (5) and PAF has been found to accumulate primarily in the portal sinusoids and not in the liver parenchyma (39), suggesting a primary interaction with sinusoidal liver cells. A further indication for the nature of the glycogenolytic signal produced by non-parenchymal cells results from experiments in which stimulation of glycogenolysis in the perfused liver by PMA, PAF and HAG was found to be blocked by indomethacin (38,40,41). Indomethacin is an inhibitor of cyclooxygenase, a key enzyme in the synthesis of prostaglandins. The blockade of glycogenolysis by indomethacin suggests that prostaglandins secreted by non-parenchymal cells, may mediate the glycogenolytic effect of PMA, PAF and HAG (Fig.

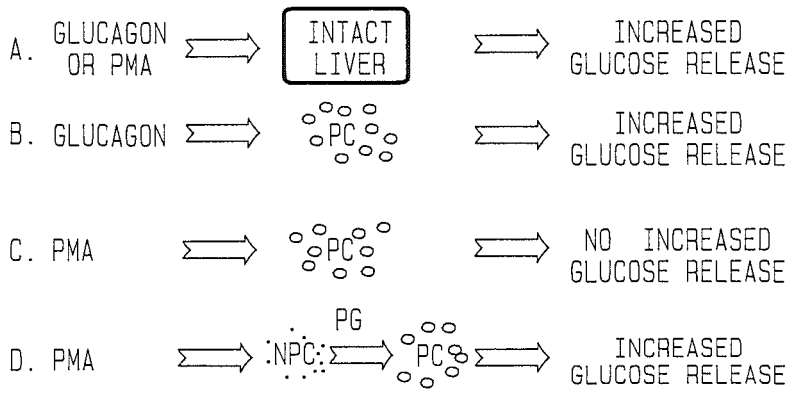

Figure 4.Mechanism of stimulation of glycogenolysis in the liver by glucagon and PMA.

PMA: phorbol-12- myristate-13-acetate; PC: parenchymal liver cells; NPC: non-parenchymal liver cells; PG: prostaglandins.

4D). Kupffer cells have been shown to produce several prostaglandins (42-44) and recently it has been demonstrated that endothelial liver cells also produce prostaglandins (45). The main prostanoid product produced by both Kupffer and endothelial liver cells in the rat is prostaglandin D_2 (45). In Kupffer cells prostaglandin D_2 accounts for 55% of the total amount of eicosanoids produced, in endothelial cells it accounts for 44%. Kupffer cells were shown to produce about 4 times as much eicosanoid (per mg cell protein) as endothelial liver cells. The most likely candidate for the glycogenolytic signal produced by non-parenchymal cells in response to PMA, PAF and HAG is therefore prostaglandin D_2.

1.4. Scope of the thesis

Glucose metabolism is an important function of the liver, and is dependent under normal conditions on both the nutritional state and hormone action. Hormones like glucagon and epinephrine act directly on parenchymal liver cells, which store glycogen. Rapid response to changes in blood glucose are effected by storing glucose as glycogen or releasing glucose from the glycogen stores. In the first part of the thesis attention is focussed on the intracellular mechanisms by which these hormones regulate the enzymes responsible for glucose metabolism.

The regulation of fructose-1,6-bisphosphatase (FBPase-1) by glucagon is greatly disputed in the literature. The rat liver enzyme has been reported to be phosphorylated (28,46,47), however, the subsequent kinetic changes are controversial (48-51). Because FBPase-1 from other sources, i.e. mouse, rabbit and ox liver, cannot be phosphorylated (46,52,53), phosphorylation is not generally accepted to play an important role in the enzyme's

regulation. An alternative suggestion is that the prevailing fructose-2,6-bisphosphate concentration in the cell determines the enzyme's activity (17). To gain more insight into the mechanism by which glucagon activates FBPase-1, the kinetic changes of the enzyme were studied in isolated hepatocytes (Appendix paper I).

Protein phosphorylation plays a crucial role in the regulation of metabolism and it has been studied extensively, however mainly in rat tissues. To extend knowledge on regulatory mechanisms of metabolism to humans; protein phosphorylation by cAMP-dependent and Ca^{2+}-dependent protein kinases in human liver was studied. Special attention was given to the influence of phosphorylated hexoses on the phosphorylation of pyruvate kinase (Appendix paper II).

The second part of the thesis concerns the mechanism of the intercellular modulation of glycogenolysis in the liver. The availability of a technique to isolate pure Kupffer and endothelial liver cells made it possible to study the effect of conditioned media of these cells on protein phosphorylation (Appendix paper III) and glycogenolysis (Appendix paper VI) in parenchymal liver cells. Furthermore the nature of the glycogenolytic signal produced by Kupffer and endothelial liver cells, which had been proposed in the literature (38,40,41) was investigated. Experiments with perfused livers were used to confirm whether the supposed mechanism, derived from studies with the isolated cell system, was operative in the intact organ (Appendix paper V).

The relevance of the mechanism of intercellular regulation for pathophysiological conditions is demonstrated in a study on the influence of endotoxin on liver glycogenolysis (Appendix

paper VI). Intercellular communication can explain the changes in glucose homeostasis associated with endotoxemia.

In summary: the studies described in this thesis were aimed at contributing to the understanding of the intracellular regulation of gluconeogenesis and glycolysis in the liver parenchymal cells. The intercellular mechanism by which glycogenolysis in the liver can be adapted to abnormal circumstances e.g. invasion of micro-organisms in the blood, is indicated.

2. RESULTS AND DISCUSSION

2.1. Regulation of fructose-1,6-bisphosphatase

Fructose-1,6-bisphosphatase catalyzes the gluconeogenic reaction in the fructose-6-phosphate/fructose-1,6-bisphosphate cycle. This cycle is considered to be one of the key points in the regulation of gluconeogenesis (17). Different mechanisms have been proposed for the regulation of fructose-1,6-bisphosphatase by glucagon. In rat liver the enzyme can be phosphorylated by cAMP-dependent protein kinase (18,46) but there is little agreement about the subsequent kinetic changes. An increase in Vmax (18), a decrease in Km (48), both an increase in Vmax and a decrease in Km (49) or no change in Km and Vmax (53) have been reported. Furthermore the relevance of the phosphorylation of the enzyme has been questioned because fructose-1,6-bisphosphatase from mouse, rabbit and ox liver cannot be phosphorylated (46,52), since these enzymes lack a C-terminal extension, containing the phosphorylation site (53).

As an alternative fructose-2,6-bisphosphate has been proposed as the factor determining the activity of fructose-1,6-bisphosphatase in response to glucagon. The level of fructose-2,6-bisphosphate is lowered in response to glucagon and thus inhibition of fructose-1,6-bisphosphatase should be relieved. In addition phosphorylated fructose-1,6-bisphosphatase is reported to be less sensitive to inhibition by fructose-2,6-bisphosphate (50,51).

In appendix paper I, a study on the mechanism by which glucagon stimulates fructose-1,6-bisphosphatase in isolated rat parenchymal liver cells, is described. Addition of glucagon to parenchymal cells leads to a 40% increase in the Vmax of fructose-1,6-bisphosphatase, without an effect on the Km (40 μM). When the

glucagon stimulated enzyme is gel-filtrated, the Vmax drops to control level. This suggests that glucagon modulates the concentration of a stimulatory factor of the enzyme. The effect of gel-filtration excludes protein phosphorylation as the cause of the increased Vmax. When the activator was added to activator-depleted enzyme, enzyme activity increased. The increase in activity was equal for glucagon-treated and control enzyme, indicating that the enzyme is equally sensitive to the activator in both glucagon-treated and control cells. The stimulation of fructose-1,6-bisphosphatase is complete within 5 min and half-maximal activation occurs at 10^{-11}M glucagon, which is well within the range of glucagon concentrations needed for other gluconeogenic effects. Activation of fructose-1,6-bisphosphatase could not be obtained with addition of dibutyryl cAMP, suggesting that glucagon stimulates the enzyme via a cAMP-independent pathway.

The data indicate that an alternative mechanism for the regulation of fructose-1,6-bisphosphatase exists, which involves the generation of an activating factor of fructose-1,6-bisphosphatase in response to glucagon.

2.2. Protein phosphorylation in human liver

Protein phosphorylation is decisive in the regulation of glucose metabolism in the liver. Phosphorylation of glycogen phosphorylase, phosphofructokinase-2/fructose-2,6-bisphosphatase and pyruvate kinase in response to glucagon, constitutes an important part of the mechanism of glucagon action (22). Most of the studies on the role of protein phosphorylation in the regulation of glucose metabolism have been performed with rat liver.

In appendix paper II, the phosphorylation of human cytosolic proteins and the influence of cAMP, Ca^{2+} and phosphorylated hexoses on the phosphorylation was studied. In this study protein phosphorylation by endogenous protein kinases was studied by adding radiolabeled ATP to human liver homogenates. Eight proteins were found to be phosphorylated by cAMP-dependent protein kinase. The major cAMP-dependent phosphoprotein was L-type pyruvate kinase. It has been reported earlier that phosphorylation by cAMP-dependent protein kinase leads to inactivation of L-type pyruvate kinase in rat liver (54,55) and human liver (56).

Micromolar concentrations of Ca^{2+} stimulated the phosphorylation of seven proteins, of which five were also stimulated by cAMP. One of the major proteins in the phosphorylation patterns, had a molecular weight of 68,000. Its phosphorylation was not stimulated by either cAMP or Ca^{2+}. So in human liver, different protein kinases are operating, i.e. cAMP-dependent, Ca^{2+}-dependent and cAMP-Ca^{2+}-independent protein kinases.

In rat liver it has been demonstrated that fructose-1,6-bisphosphate, an effective allosteric inhibitor of pyruvate kinase, inhibits the phosphorylation of this glycolytic enzyme (57,58). Fructose-2,6-bisphosphate, a potent allosteric activator of phosphofructosekinase-1, enhances the phosphorylation of phosphofructokinase-1 and inhibits the phosphorylation of phosphofructokinase-2/fructose-2,6-bisphosphatase (59,60).

In human liver, fructose-1,6-bisphosphate, and to a lesser extent glucose-1,6-bisphosphate inhibited the phosphorylation of L-type pyruvate kinase, similar to the situation in rat liver. Fructose-6-phosphate, glucose-6-phosphate and fructose-2,6-bisphosphate were without effect on the phosphorylation of L-type pyruvate kinase from human liver.

Besides the phosphorylation of pyruvate kinase, the phosphorylation of the Mw 68,000 protein was also influenced by phosphorylated hexoses. Its phosphorylation was inhibited by fructose-1,6-bisphosphate, glucose-1,6-bisphosphate, fructose-6-phosphate and glucose-6-phosphate but not by fructose-2,6-bisphosphate. The data suggest an important role for phosphorylated hexoses in the regulation of the phosphorylation of both pyruvate kinase and the Mw 68,000 protein.

It can be concluded that in human liver, protein phosphorylation is regulated by cAMP, Ca^{2+} and phosphorylated hexoses, a situation similar to that found in rat liver.

2.3. Influence of Kupffer and endothelial liver cells on protein phosphorylation and glycogenolysis in parenchymal liver cells.

Protein phosphorylation in the liver has been the subject of many investigations. Several important enzymes of major metabolic routes in the liver have been shown to be regulated by phosphorylation/dephosphorylation. In most studies isolated parenchymal liver cells were used to demonstrate the influence of several hormones on protein phosphorylation. Besides parenchymal cells, other cell types are present in the liver and of these cells the Kupffer cells are good candidates to influence parenchymal cell metabolism, since it is known that these cells produce several prostaglandins (42-44). The intermediatory role for non-parenchymal liver cells in the stimulation of glycogenolysis by phorbol ester, platelet activating factor and heat aggregated immunoglobulin G has been proposed (38,40). The availability of a system to isolate pure Kupffer and endothelial cells made it possible to study the influence of conditioned media of isolated Kupffer and endothelial liver cells on protein phosphorylation in parenchymal

cells, as described in Appendix paper III. Kupffer and endothelial liver cells were isolated and incubated for 1 hour, then conditioned media were collected. Parenchymal cells were incubated with radiolabeled phosphate, and after a equilibration time of 1 hour, conditioned media from Kupffer and endothelial cells were added. Conditioned media of Kupffer and endothelial cells both increased the phosphorylation state of a Mw 97,000 and a Mw 47,000 protein. The phosphorylation state of a Mw 63,000 protein was decreased. These effects could be mimicked by prostaglandins E_1, E_2 and D_2. The identity of two of the influenced phosphoproteins could be deduced. The molecular weight of one of these proteins corresponds to the molecular weight of glycogen phosphorylase. The activity of glycogen phosphorylase is known to be regulated by phosphorylation, therefore the influence of conditioned media and prostaglandins on the activity of glycogen phosphorylase was determined. Phosphorylase activity in parenchymal cells was stimulated by Kupffer and endothelial cell media and prostaglandins E_1, E_2 and D_2, indicating that the Mw 97,000 protein could indeed be phosphorylase.

The Mw 63,000 protein of which the phosphorylation was negatively influenced by both media and prostaglandins, could be identified by the fact that it was secreted as a phosphoprotein and has a pI of 5.0-5.6. Such a secretory phosphoprotein has recently been described (61) as a negatively regulated acute phase protein. The synthesis of this protein is depressed during acute phase response (62) and our data suggest that prostaglandins secreted by Kupffer and/or endothelial liver cell might play a role in the regulation of this process.

From the data it can be concluded that Kupffer and endothelial cells can influence protein phosphorylation in parenchymal

cells and thereby might influence the metabolic response of these cells. This was tested in appendix paper IV and it was found that glycogenolysis in parenchymal cells was stimulated by both Kupffer (140%) and endothelial liver cell media (127%). The separation of the secretory products of Kupffer and endothelial liver cells in a low and a high molecular weight fraction, indicated that the active factor(s) had a low molecular weight. Since both Kupffer and endothelial liver cells are known to produce prostaglandins (42-44), prostanoid free media were prepared by incubating Kupffer and endothelial liver cells with acetylsalicylic acid. These prostanoid free media had no effect on glycogenolysis in parenchymal cells, indicating that the active factor(s) present in Kupffer and endothelial liver cell media is (are) of prostanoid nature.

The main prostanoid product of both Kupffer and endothelial liver cells is reported to be prostaglandin D_2 (45). In Kupffer cells prostaglandin D_2 accounts for 55% of the total amount of eicosanoids produced; in endothelial liver cells it accounts for 44%. Therefore the effect of prostaglandin D_2 on the glycogenolysis in parenchymal cells was studied. Prostaglandin D_2 stimulated glucose secretion of parenchymal cells up to 70%, while the stimulation by prostaglandin E_1 and E_2 was 20% and 30% respectively.

The data indicate that Kupffer and endothelial liver cells can modulate glycogenolysis in parenchymal liver cells by increasing the phosphorylation state of phosphorylase. Prostaglandins, in particular prostaglandin D_2, may mediate the intercellular communication.

2.4. Intercellular communication in the perfused liver system.

Stimulation of glycogenolysis in perfused liver by PMA, HAG and PAF can be blocked by indomethacin (38,40,41), suggesting that prostaglandins may be involved in this stimulation. Because non-parenchymal cells are very active in the production of prostaglandins and the main prostaglandin produced by Kupffer and endothelial liver cells is prostaglandin D_2, it was verified if changes in prostaglandin D_2 could mediate the stimulation of glycogenolysis in perfused liver by PMA.

In appendix paper V it is shown that prostaglandin D_2 production by the liver is more than doubled in response to PMA, a process paralleled by the increase in glycogenolysis. Both responses have a lag time of about 5 min. Infusion of prostaglandin D_2 in the liver immediately results in an increased glycogenolysis. So apparently PMA increases, after a lag time, the prostaglandin D_2 production and the time course of the stimulation of glycogenolysis is consistent with a mediating role of prostaglandin D_2. In contrast to prostaglandin D_2, prostaglandin E_2 has been reported to be uninfluenced by PMA (37). In the literature it has been hypothesized that glycogenolysis is influenced by haemodynamic effects that occur in response to PMA (37). This hypothesis was necessary because no effect of PMA on prostaglandin production in the liver was known and even an absence of any effect on prostaglandin E_2 production was published (37). The data in appendix paper IV and V show that prostaglandin D_2 production is increased by PMA and can stimulate glycogenolysis in parenchymal cells. An additional effect of hypoxia caused by the haemodynamic effects associated with PMA stimulation is therefore not necessary to explain the metabolic response.

Endotoxin, a bacterial toxin, is removed from the circulation by Kupffer cells (63,64). Since endotoxemia is associated with changes in glucose homeostasis, the influence of endotoxin on glycogenolysis in the liver was studied (appendix paper VI). It was found that endotoxin stimulates glycogenolysis in the perfused liver but fails to do so in isolated parenchymal cells. Furthermore, the stimulation of glycogenolysis by endotoxin can be blocked by acetylsalicylic acid, indicating that prostaglandins may be involved in the effect of endotoxin on glycogenolysis. To verify this point the influence of endotoxin on the production of prostaglandin D_2 by the liver was studied. It was found that endotoxin stimulated the production of prostaglandin D_2 five-fold, indicating that endotoxin may act via the induction of prostaglandin D_2 production in non-parenchymal liver cells. Endotoxin has been reported to stimulate eicosanoid production in various cells including vascular endothelial cells, neutrophils and preoptic nerve cells (65,66) and appears to act via protein kinase C (67), the intracellular target for PMA. It is likely that in the liver endotoxin acts on glycogenolysis via the same intercellular mechanism as PMA. Stimulation of glycogenolysis by endotoxin via intercellular communication may explain the hyperglycemia observed in early or mild endotoxemia (64).

The intercellular mechanism of activation of glycogenolysis, in which prostaglandin D_2, produced by non-parenchymal liver cells interacts with parenchymal cells, leading to activation of glycogen phosphorylase, may operate under certain pathophysiological conditions e.g. endotoxemia adds a new mechanism to the complex regulation of glucose homeostasis by the liver.

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APPENDIX PAPER I

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Mechanism of glucagon stimulation of fructose-1,6-bisphosphatase in rat hepatocytes

Involvement of a low-M_r activator

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Isolated rat hepatocytes were incubated in the absence or presence of glucagon and the activity of fructose-1,6-bisphosphatase was measured in cell extracts. After glucagon treatment the V_{max} was increased (20–50%) whereas the K_m remained unchanged. The stimulation was complete at 5 min after addition of glucagon. The glucagon concentration needed for maximal stimulation was 10^{-9} M. After gel filtration the fructose-1,6-bisphosphatase activity in extracts of glucagon-treated cells was lowered to the control level. The effect of glucagon could not be completely mimicked by dibutyryl cAMP. The data indicate that in addition to the possible regulatory role of enzyme phosphorylation, a positive effector is involved in the stimulation of fructose-1,6-bisphosphatase activity by glucagon.

Fructose-1,6-bisphosphatase Glucagon Low-M_r activator (Rat hepatocyte)

1. INTRODUCTION

Fructose-1,6-bisphosphatase (EC 3.1.3.11; FBPase) is part of the regulatory important fructose 1,6-bisphosphate/fructose 6-phosphate substrate cycle, and is thought to be regulatory for the gluconeogenic/glycolytic pathway [1]. Rat liver FBPase can be phosphorylated in vitro by cAMP-dependent protein kinase [2,3] and in hepatocytes its phosphorylation is increased by glucagon [4]. FBPases from mouse, rabbit and ox liver as well as from pig kidney cannot be phosphorylated [3,5], since they lack a C-terminal extension, containing the phosphorylation site [6]. The effect of phosphorylation on the kinetic properties of the enzyme is under dispute. An increase in V_{max} [2], a decrease in K_m [7], both an increase in V_{max} and a decrease in K_m [8] or no change in V_{max} and K_m [2,6] have been reported. Phosphorylated FBPase has also been reported to be less sensitive to inhibition by fructose 2,6-bisphosphate than the unphosphorylated enzyme [9,27]. Phosphorylation of FBPase is not generally accepted as playing an important role in the regulation of gluconeogenesis and glycolysis [10].

Allosteric regulation of FBPase can be performed by several metabolites. FBPase is inhibited by AMP [11] and fructose 2,6-bisphosphate [12,13]. Since the fructose 2,6-bisphosphate level in hepatocytes is lowered after glucagon treatment [14,15], fructose 2,6-bisphosphate has been put forward as the main factor controlling the activity of FBPase in vivo [10] through a relief of enzyme inhibition. However, Corredor et al. [16] found that micromolar concentrations of fructose 2,6-bisphosphate can also stimulate FBPase activity.

Administration of glucagon in vivo leads to increased activity of rat [17,18] and mouse [19] liver FBPase. Since mouse liver FBPase cannot be

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phosphorylated [3,6] it is unlikely that phosphorylation plays a decisive role in determining the activity of FBPase.

Here, we show that treatment of hepatocytes with glucagon leads to a rapid increase in V_{max} of FBPase. This increase is not due to phosphorylation of the enzyme or to a change in fructose 2,6-bisphosphate concentration, but is caused by a low-M_r activator.

2. MATERIALS AND METHODS

Male Wistar rats (250 g) were anesthetized with 18 mg Nembutal given intraperitoneally. Parenchymal liver cells were isolated by perfusion with collagenase by the method of Seglen [20]. 2% albumin was added to the collagenase buffer and washing buffer. Cells were incubated in Krebs-Ringer with a protein concentration of 10 mg/ml. The cells were kept in suspension by shaking in a water bath at 37°C and gassed with 95% O_2, 5% CO_2. Viability of the cells was usually over 90% as judged by phase-contrast microscopy. Incubations were stopped by cooling in ice and after addition of 1 mM 2-mercaptoethanol samples were immediately homogenized. Homogenates were centrifuged for 10 min at 10000 × g. In the supernatant, FBPase activity was assayed immediately after centrifugation and an Aminco DW2 double-beam spectrophotometer was used to monitor the assay at 340/400 nm. The assay mixture consisted of 10 mM potassium phosphate buffer (pH 7.5), 25 mM 2-mercaptoethanol, 1 mg/ml albumin, 2.5 mM $MgSO_4$, 0.4 mM NADP, 7 units glucose-6-phosphate dehydrogenase and 3.5 units phosphohexose isomerase (both enzymes were desalted on Sephadex G-25); final volume 2.3 ml. The mixture was preincubated with 200 μl sample for 3 min at 30°C and the reaction initiated by the addition of 100 μl fructose 1,6-bisphosphate solution. In the samples some 6-phosphogluconate dehydrogenase activity was present, but it was verified that under the present conditions it did not interfere with our measurements. The FDPase activity was determined between 1 and 3 min after starting the reaction, when V was nearly constant. Where indicated, aliquots of the supernatant fraction were desalted on Sephadex G-25 medium (equilibrated with H_2O) by the method of Penevsky [21]. The low-M_r fraction was obtained by elution and subsequent lyophilisation. Protein concentrations were determined by the method of Lowry et al. [22]. L-type pyruvate kinase activity was determined as in [23]. Collagenase type I, bovine serum albumin fraction V, fructose 1,6-bisphosphate and fructose 2,6-bisphosphate were from Sigma.

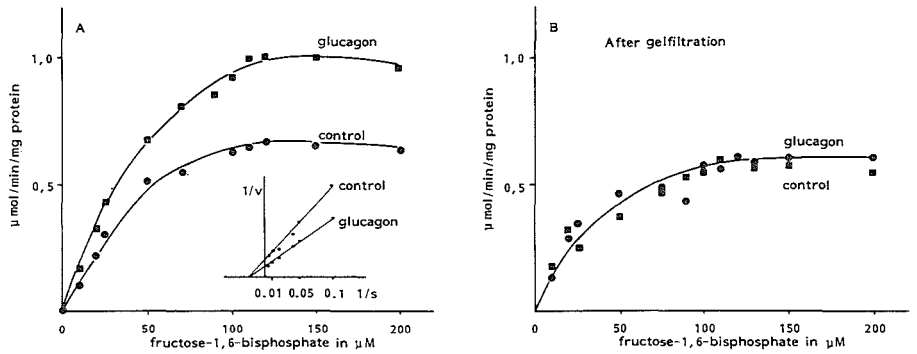

Fig.1. Effect of glucagon on the FBPase activity. FBPase activity was measured in supernatants of control (●) and glucagon (10^{-7} M, 10 min) treated cells (■), before (A) and after (B) gel filtration. Inset: double-reciprocal plot of substrate curves of control and glucagon stimulated FBPase. Results shown are from a typical experiment (n = 7).

3. RESULTS AND DISCUSSION

Substrate curves of FBPase from control and glucagon-stimulated parenchymal cells are given in fig.1A. After glucagon treatment the activity of FBPase is increased. The double-reciprocal plot indicates that the V_{max} is increased by glucagon treatment whereas the K_m (40 μM) remains unchanged. Routinely we found 20–40% stimulation of FBPase upon glucagon addition, although occasionally up to 70% stimulation was observed. To determine whether the increase in activity is caused by covalent modification or by the presence of an effector, the FBPase activity was determined in gel-filtered samples (fig.1B). Upon gel filtration the activity of control samples was unchanged, while the activity in the glucagon-treated samples was lowered to the control level. This indicates that the increase in FBPase activity after glucagon treatment is not caused by phosphorylation but probably mediated by an effector.

In our experiments however, an increased phosphorylation state of FBPase was indicated by the finding that in gel-filtered glucagon-treated samples FBPase was less sensitive to fructose 2,6-bisphosphate inhibition than control FBPase, similar to the findings in [9]. Since the activation of FBPase by glucagon can be abolished by gel filtration, a low-M_r activator is suspected as being responsible for the observed difference. Readdition of the low-M_r fraction from glucagon-treated samples indeed leads to an increase in FBPase activity (table 1). The nature of this activator is however unclear. Activation of FBPase cannot be explained by decreased inhibition by fructose 2,6-bisphosphate since gel filtration would then lead to increased FBPase activity of the control samples. Although we found, as did Corredor et al. [16], that 1 μM fructose 2,6-bisphosphate could stimulate FBPase after gel filtration, fructose 2,6-bisphosphate could not be responsible for activating FBPase after glucagon treatment because the concentrations of fructose 2,6-bisphosphate in the assay can be calculated to be about 2 nM for glucagon-treated and 20 nM for control samples, which are too low to stimulate FBPase. Calculations were based on data from [24]. Moreover, in the presence of fructose 2,6-bisphosphate at inhibiting concentrations, added low-M_r fraction stimulates the FBPase activity, indicating that the activator is not fructose 2,6-bisphosphate. To characterize further the kinetics of the stimulation of FBPase activity by glucagon we studied the dose and time dependency of the effect. Fig.2 shows that the effect is almost complete at 5 min after addition of glucagon. Fig.3 indicates that 10^{-9} M glucagon is needed for maximal activation of FBPase activity, while half-maximal activation occurs at about 10^{-11} M, which is well within the range of the glucagon dose needed for other gluconeogenic effects [10].

Since the glucagon effect on gluconeogenesis can be mimicked by dibutyryl cAMP [10], we compared the effect of dibutyryl cAMP with that of glucagon. Although dibutyryl cAMP was equally active as glucagon in inactivating L-type pyruvate kinase [25], we observed only a marginal effect of dibutyryl cAMP on FBPase (table 2). This implies

Table 1

Effect of low-M_r fraction on the activity of FBPase

	Cells incubated without glucagon		Cells incubated with glucagon	
	FBPase (pmol/min per mg protein)	% stimulation	FBPase (pmol/min per mg protein)	% stimulation
Control	553 ± 51	–	519 ± 31	–
20 μl activator	623 ± 33	13	589 ± 39	14
40 μl activator	666 ± 9[a]	21	671 ± 15[a]	29

[a] Significant difference from control ($P < 0.05$, Student's t-test, tested for equal variances)

Low-M_r fraction ('activator') was isolated and concentrated from a glucagon-stimulated sample. Different amounts (20, 40 μl) were added to assays of untreated and glucagon-treated gel-filtered samples. Values are given ± SD ($n = 3$)

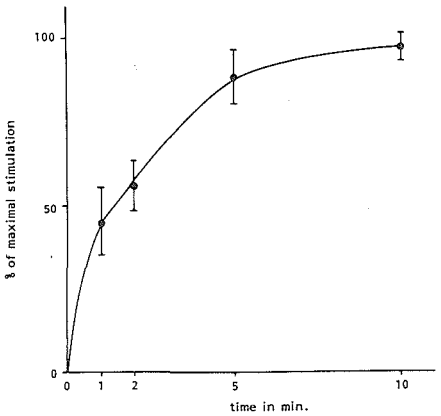

Fig.2. Time dependency of the FBPase activation by glucagon. FBPase activity was measured at 100 μM fructose 1,6-bisphosphate, in supernatants of cell stimulated with glucagon (10^{-7} M) for different periods of time. Values are given ± SE (n = 4).

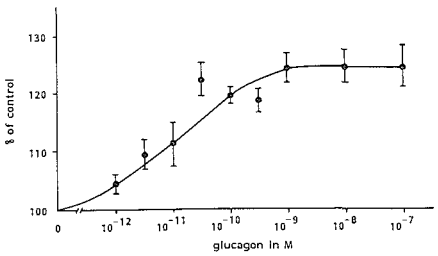

Fig.3. Dose dependency of the FBPase activation by glucagon. FBPase activity was measured at 100 μM fructose 1,6-bisphosphate in supernatants of cells stimulated with different doses of glucagon for 10 min. Values are given ± SE (n = 5).

that besides cAMP other second messengers might be involved, perhaps Ca^{2+}, which is known to increase after glucagon treatment [26]. The difference in the effects of glucagon and dibutyryl cAMP is a further indication against the involvement of cAMP-dependent phosphorylation or fructose 2,6-bisphosphate in the activation of FBPase in rat hepatocytes. Our data indicate that a low-M_r activator is involved in the activation of FBPase by glucagon in rat hepatocytes.

Table 2

Influence of glucagon and dibutyryl cAMP on the fructose-1,6-bisphosphatase and L-type pyruvate kinase activity

	FBPase (pmol/min per mg protein)	% stimulation	Pyruvate kinase (v/V_{max})	% inhibition
Control	545 ± 7	–	0.66 ± 0.10	–
Glucagon	675 ± 6[a]	24	0.34 ± 0.09[a]	51
Dibutyryl cAMP	581 ± 27	7	0.33 ± 0.07[a]	50

[a] Significant difference from control ($P < 0.01$, Student's t-test tested for equal variances)

FBPase and pyruvate kinase activity were measured in supernatants of cells stimulated with glucagon (10^{-7} M) or dibutyryl cAMP (10^{-4} M) for 10 min. FBPase activity was measured at 100 μM fructose 1,6-bisphosphate. Pyruvate kinase was measured at 2 mM phospho*enol*pyruvate in the absence (v) and presence (V_{max}) of 50 μM fructose 1,6-bisphosphate. Values are given ± SD (n = 4)

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APPENDIX PAPER II

PHOSPHORYLATION OF HUMAN LIVER CYTOSOLIC PROTEINS: INFLUENCE OF cAMP, Ca^{2+} AND PHOSPHORYLATED HEXOSES.

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ABBREVIATIONS

cAMP - adenosine 3', 5'-monophosphoric acid

Fru-2,6-P_2 - fructose-2,6-bisphosphate

Fru-1,6-P_2 - fructose-1,6-bisphosphate

Glu-1,6-P_2 - glucose-1,6-bisphosphate

Fru-6-P - fructose-6-phosphate

Glu-6-P - glucose-6-phosphate

PEP - phosphoenolpyruvate

Enzymes:

Pyruvate kinase (EC 2.7.40).

SUMMARY

In order to verify the relevance of findings on the phosphorylation of rat liver cytosolic proteins for the human situation, the phosphorylation of human liver cytosolic proteins by endogenous human protein kinase was studied, whereby the relative role of cAMP-dependent, Ca^{2+}-activated and cAMP-independent protein kinases was taken into account. Heat-stable inhibitor of cAMP-dependent protein kinase inhibits the phosphorylation of eight proteins with a major effect on L-type pyruvate kinase. Ca^{2+} in the micromolar range stimulates the phosphorylation of seven proteins with a most prominent effect on phosphorylase. Of two proteins the phosphorylation was specifically stimulated by Ca^{2+}, whereas the other five were also influenced by cAMP-dependent protein kinases. A prominent protein with a M.W. of 68.000 was phosphorylated by a cAMP and Ca^{2+}-independent pathway. However its phosphorylation was completely blocked by physiological concentrations of phosphorylated hexoses. It is concluded that in human liver the phosphorylation of cytosolic proteins is regulated by cAMP, Ca^{2+} and phosphorylated hexoses, in a comparable way as in rat liver suggesting that hormonal regulation of glucose homeostasis by human liver depends on the complex interplay of various types of protein kinase. It is suggested that application of the applied phosphorylation system, in which only 10 µl of a 20% homogenate supernatant is needed, for patients with problems in the regulation of of glucose homeostasis may form a rapid screening method in order to analyse the molecular basis of their disturbed metabolism.

INTRODUCTION

Glycolysis and gluconeogenesis are well regulated metabolic processes (1). Hormonal control of these metabolic routes occurs through changes in the activity of the enzymes which catalyze key reactions. These changes are mainly brought about by phosphorylation and dephosphorylation of these enzymes and/or by changes in the concentration of allosteric effectors. Phosphorylation may occur by cAMP-dependent, Ca^{2+}-activated or cAMP-independent protein kinases while phosphorylation rates may in addition be influenced by metabolites (1). A major glycolytic enzyme regulated by phosphorylation and allosteric effectors is L-type pyruvate kinase. Pyruvate kinase type-L in rat (2,3) and human (4) was shown to be phosphorylated by cAMP-dependent protein kinase, resulting in its inactivation. Fru-1,6-P_2, an effective allosteric activator of L-type pyruvate kinase (5), inhibits the phosphorylation (6). Another phosphorylated hexose, involved in metabolic regulation is Fru-2,6-P_2, a potent allosteric activator of 6-phosphofructo-1-kinase (7). Fru-2,6-P_2 enhances the phosphorylation of 6-phosphofructo-1-kinase and inhibits the phosphorylation of 6-phosphofructo-2-kinase/-fructose-2,6-bisphosphatase (8).

Since an important role of phosphorylation is obvious from animals studies, it is relevant to determine if similar processes are operative in human liver. In this paper we describe the effects of cAMP, Ca^{2+} and phosphorylated hexoses on the phosphorylation of cytosolic proteins from human liver catalysed by endogenous human protein kinases.

MATERIALS AND METHODS

Preparation of soluble fraction

Fresh human liver samples, available from medically indicated liver biopsies, were immediately frozen in liquid N_2 and stored at -70°C until use. A 20% (w/v) homogenate was made in 250 mM sucrose, 25 mM Tris/HCl (pH 7.5) and 2 mM β-mercapto ethanol. After centrifugation for 6 min. at 30 psi in an airdriven ultracentrifuge (Beckman) the supernatant was desalted by the method of Penesky (9) on Sephadex G-25-medium, equilibrated with 25 mM Tris/HCl (pH 7.5) and 2 mM β-mercaptoethanol. This soluble fraction was preincubated for 30 min. at 20°C with 5 mM $MgCl_2$, in order to dephosphorylate proteins prior to the phosphorylation experiments.

Phosphorylation experiments

Soluble fraction (10 µl) was incubated for 5 min. with 10 µl of a mixture containing 200 mM KCl, 25 mM Tris/HCl (pH 7.5), 40 mM phosphate, 10 mM theophylline and 5 mM $MgCl_2$ and with 5 µl containing the additions indicated in the legends of the figures. Phosphorylation was started by adding 5 µl 600 µM [γ-^{32}P]ATP(1 Ci/mmole) + 5 mM $MgCl_2$. The reaction was stopped by adding 15 µl of a mixture containing 62 mM Tris/-H_3PO_4 (pH 6.8), 12,5% glycerol (v/v), 1,25% sodium dodecyl sulphate (w/v), 2,5% β-mercaptoethanol (v/v) and immediately heated for 5 min at 95°C.

Separation of proteins

Proteins were separated by SDS-PAGE on 10% gels (10). After drying under vacuum the gels were exposed to SB-5 auto-

radiography films (Kodak), exposure times were chosen to assure linearity between the amount of radioactivity and optical density of the film. The phosphorylation patterns were analysed with a densitometer (Vitatron TLD 100) and phosphorylation bands were quantified by measuring peak heights in the densitograms.

Chemicals

Anti human Albumin coupled to CNBr-activated Sepharose 4B (Pharmacia) was used to remove Albumin from the soluble fraction. Fru-2,6-P_2 was from Sigma, the other phosphorylated hexoses were from Boehringer. [γ-^{32}P]ATP was from the Radiochemical Centre, Amersham. Protein kinase inhibitor was from Sigma.

RESULTS AND DISCUSSION

In order to identify substrates from cAMP-dependent and Ca^{2+}-dependent protein kinase, in human liver cytosol, the phosphorylation of cytosolic proteins by endogenous protein kinases was studied in the absence and presence of cAMP or Ca^{2+}. To establish the role of cAMP-dependent protein kinase unequivocally protein kinase inhibitor, which inhibits specifically the catalytic subunit of cAMP-dependent protein kinase, was added to the assay medium in excess (11). Careful inspection of the autoradiograph (Fig. 1) shows that eight phosphorylation bands are specifically influenced by this addition of which pyruvate kinase is the most prominent band (lane d vs e). Addition of 10 μM Ca^{2+} stimulates the ^{32}P incorporation into seven proteins of which phosphorylase is

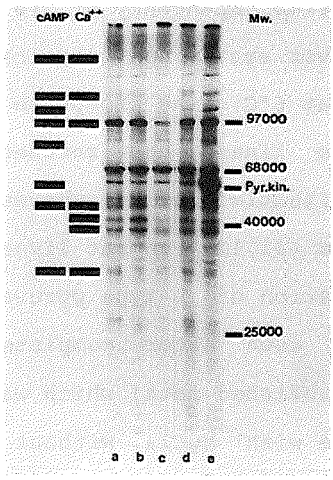

Figure 1.

The effect of cAMP and Ca^{2+} on the phosphorylation of human liver cytosolic proteins.
Phosphorylation of samples preincubated with $MgCl_2$, took place at 20°C for 5 min. Additions were: a: none; b: 10 µM Ca^{2+}; C: 100 µM EGTA; d: 10 µg/ml protein kinase inhibitor; e: 10µM cAMP. Bars indicate stimulation of phosphorylation of a protein by cAMP or Ca^{2+}.

most prominent. Five of these proteins are also phosphorylated in a cAMP-dependent way. In addition to cAMP-dependent and Ca^{2+}-dependent phosphorylation, in rat liver endogenous protein kinases are present of which the activity is independent of these two effectors. In human liver one prominent protein with a M.W. of 68.000 is observed of which its phosphorylation is independent of Ca^{2+} or cAMP.

In order to determine effects of phosphorylated hexoses

on the maximal extent of phosphorylation of human L-type pyruvate kinase the time dependency of its phosphorylation in the present human liver system was examined. Figure 2 shows that after 5 min. at 6°C, the phosphate incorporation into human L-type pyruvate kinase has reached a plateau value comparable to the situation in rat, whereafter 2 min. a plateau value is reached (11,12). In rat liver inhibition of the level of phosphorylation of L-type pyruvate kinase by Fru-1,6-P_2 could only be seen after complete dephosphorylation (8; Casteleijn, unpublished data) which was achieved by preincubating the enzyme with $MgCl_2$. Without such a preincubation also in human liver only a marginal inhibition of the

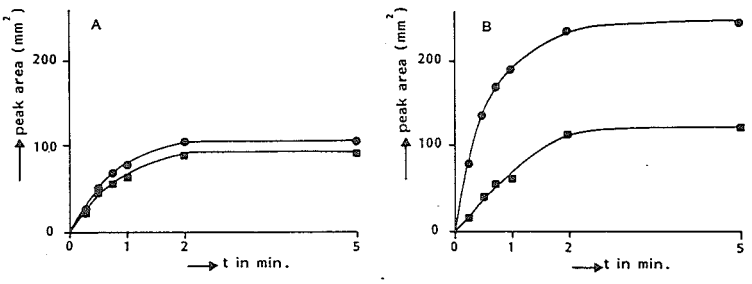

Figure 2.

Effect of Fru-1,6-P_2 on the time-dependent phosphorylation of L-type pyruvate kinase in (not) preincubated samples.

Phosphorylation was carried out at 6°C in the presence of 10 µM cAMP, in the absence (●) or presence (■) of 100 µM Fru-1,6-P_2 on samples preincubated (B) with 5 mM $MgCl_2$ for 30' at 20°C or samples that were not preincubated (A). Phosphorylation is expressed as peak height from the densitograms made from the autoradiographs of the SDS-PAGE gels.

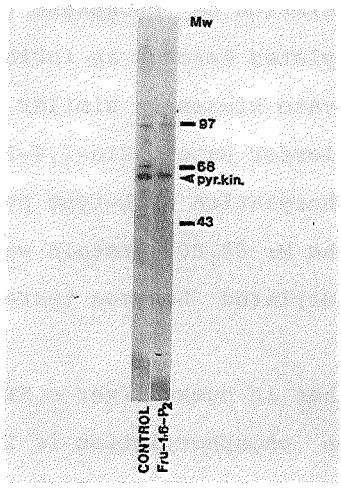

Figure 3.

Effects of Fru-1,6-P_2 on the phosphorylation of human cytosolic proteins.

Phosphorylation of samples preincubated with $MgCl_2$ took place at 20°C for 5 min. with or without 100 µM Fru-1,6-P_2.

phosphorylation of pyruvate kinase by Fru-1,6-P_2 is noticed, stressing the relevance of prior dephosphorylation before performing the phosphorylation experiments.

In Figure 3 is indicated that Fru-1,6-P_2 not only influences the extent of phosphorylation of pyruvate kinase but also abolishes the cAMP, Ca^{2+}-independent phosphorylation of a 68.000 M.W. protein.

A comparison was made between the effects of different phosphorylated hexoses on the extent of phosphorylation of

pyruvate kinase and the Mr 68.000 protein. These experiments were performed with the preincubated samples to assure maximal possible incorporation of phosphate (Fig. 4). The specificity of phosphorylated hexoses as inhibitors of the phosphorylation of pyruvate kinase is similar to rat liver. Only Fru-1,6-P_2 and to a lesser extent Glu-1,6-P_2, are capable of inhibiting the phosphorylation of L-type pyruvate kinase. The phosphorylation of the Mr 68.000 protein was completely inhibited by all phosphorylated hexoses tested, except by Fru-2,6-P_2.

This indicates that in human liver a Mr 68.000 protein is present of which the phosphorylation is independent of Ca^{2+} or cAMP but is specifically regulated by phosphorylated he-

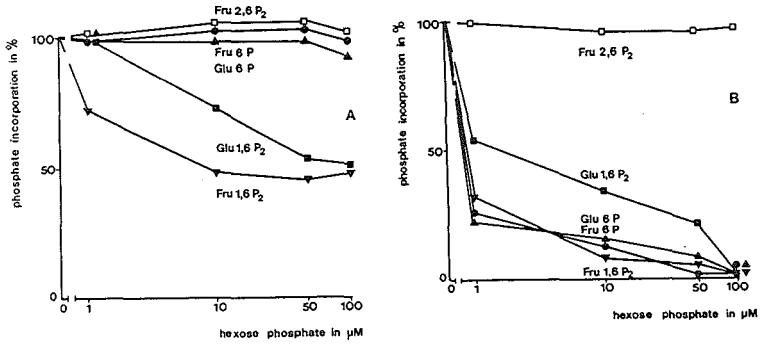

Figure 4.

Inhibition of the phosphorylation of L-type pyruvate kinase (A) and a Mr 68.000 protein (B) by various phosphorylated hexoses. Phosphorylation of samples preincubated with $MgCl_2$ was carried out for 5 min. at 20°C, in the presence of different concentrations of Fru-1,6-P_2 (▼), Fru-2,6-P_2 (□), Fru-6-P (●), Glu-1,6-P_2 (■) or Glu-6-P (▲). Phosphorylation was expressed as percentage of phosphate incorporation in the absence of phosphorylated hexoses. The data are based on two separate experiments with liver samples from different patients.

xoses. The effects of phosphorylated hexoses on the phosphorylation of L-type pyruvate kinase in human liver are similar to earlier observations in rat liver. These data suggest that in human liver phosphorylated hexoses may play an important role in determining the phosphorylation state of these two proteins.

In conclusion our data indicate that in human liver the phosphorylation of cytosolic proteins is regulated by cAMP, Ca^{2+} and phosphorylated hexoses, in a comparable way as in rat liver, suggesting that hormonal regulation of glucose homeostasis by human liver depends on the complex interplay of various types of protein kinases.

The phosphorylation system as used in the present study allows the determination of the rate of phosphorylation and the maximal extent of phosphorylation of a range of proteins as catalysed by endogenous protein kinases, whereby small amounts of liver samples (20 µl of a 20% homogenate supernatant) are needed.

Application of the present system to liver biopsies from patients with problems in the regulation of glucose homeostasis may form a rapid screening method in order to learn more on the molecular basis of their disturbed metabolism.

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APPENDIX PAPER III

Conditioned media of Kupffer and endothelial liver cells influence protein phosphorylation in parenchymal liver cells

Involvement of prostaglandins

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The possible role of Kuppfer and endothelial liver cells in the regulation of parenchymal-liver-cell function was assessed by studying the influence of conditioned media of isolated Kuppfer and endothelial cells on protein phosphorylation in isolated perenchymal cells. The phosphorylation state of three proteins was selectively influenced by the conditioned media. The phosphorylation state of an M_r-63000 protein was decreased and the phosphorylation state of an M_r-47000 and an M_r-97000 protein was enhanced by these media. These effects could be mimicked by adding either prostaglandin E_1, E_2 or D_2. Both conditioned media and prostaglandins stimulated the phosphorylase activity in parenchymal liver cells, suggesting that the M_r-97000 phosphoprotein might be phosphorylase. Parenchymal liver cells secrete a phosphoprotein of M_r-63000 and pI 5.0–5.5. The phosphorylation of this protein is inhibited by Kuppfer- and endothelial-liver-cell media, and prostaglandins E_1, E_2 and D_2 had a similar effect. The data indicate that Kuppfer and endothelial liver cells secrete factors which influence the protein phosphorylation in parenchymal liver cells. This forms further evidence that products from non-parenchymal liver cells, in particular prostaglandin D_2, might regulate glucose homoeostasis and/or other specific metabolic processes inside parenchymal cells. This stresses the concept of cellular communication inside the liver as a way by which the liver can rapidly respond to extrahepatic signals.

INTRODUCTION

Protein phosphorylation is a well-studied regulatory phenomenon in the liver. Many key enzymes of major metabolic routes in the liver are shown to be regulated by phosphorylation/dephosphorylation. So far, attention has been focused on the major liver cell type, parenchymal cells, and several hormones have been shown to regulate the phosphorylation/dephosphorylation status in these cells [1,2].

However, besides parenchymal cells, the liver contains other cell types, i.e. Kupffer, endothelial, fat-storing and pit cells [3]. Although knowledge of the secretory response of the non-parenchymal liver cells is limited, it is known that Kupffer cells are able to produce various prostaglandins [4–6]. It has even been suggested, on the basis of studies with perfused liver [7–10], that phorbol esters and platelet-activating factor might exert a regulatory effect on parenchymal liver cells mediated by non-parenchymal cells. This hypothesis was based on the observation that these agents do not stimulate glycogenolysis in isolated parenchymal liver cells [10–14], whereas in the perfused liver system a stimulation of glycogenolysis [7–10] by these agents could be blocked by indomethacin [7,10,15,16], a cyclo-oxygenase inhibitor. Previously we described that prostaglandin D_2 concentrations in liver perfusates are increased by phorbol ester, and it was shown that prostaglandin D_2 could stimulate glucose release from the liver [17]. However, no direct evidence with isolated cells was obtained, which showed that secretory products of non-parenchymal cells can exert a regulatory response in parenchymal liver cells. In order to test directly the possibility of cellular communication in the liver, we isolated the various cell types and studied the influence of conditioned media of Kupffer and endothelial cells on the phosphorylation state of parenchymal-cell proteins. The effects of these media were compared with the effect of prostaglandins.

Parenchymal liver cells have been shown to synthesize and secrete a phosphorylated acute-phase protein [18]. This protein has an M_r of 63000 and a pI of 4.8–5.3, and its synthesis is depressed during acute-phase response [19]. The influence of conditioned media of Kupffer and endothelial liver cells on the phosphorylation of this secretory phosphoprotein was also studied.

MATERIALS AND METHODS

Prostaglandins E_1, E_2 and D_2 collagenase type I and IV were from Sigma; carrier-free [^{32}P]P_i was from Amersham; other chemicals were of P.A. quality.

Male Wistar rats, fed *ad libitum*, weighing 200–220 g were used. Nembutal (18 mg) was given intraperitoneally for anaesthesia, usually performed between 09:00 and 10:00 h.

Parenchymal liver cells were isolated after perfusion

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for 20 min with collagenase (type IV, 0.1%) by the method of Seglen [20], modified as previously described [21]. Parenchymal liver cells were incubated at 37 °C under constant shaking at 5 mg of cell protein/ml in phosphate-free Krebs–Ringer bicarbonate buffer, saturated with O_2/CO_2 (19:1), pH 7.4, keeping the viability of the cells > 95%. At zero time 1 mCi of [^{32}P]P_i/ml was added, and the cells were preincubated for 1 h. During the preincubation time the [^{32}P]P_i equilibrated and the specific radioactivity of [^{32}P]ATP reached a steady state [22]. Cells were challenged for 5 min with non-parenchymal-cell media or prostaglandins. Subsequently 50 μl of cell suspension was added to 200 μl of a digitonin (2 mg/ml)-containing buffer as described in [23]. After 20 s the samples were centrifuged (30 s, 10000 *g*) and 50 μl of the resulting supernatant was mixed with 200 μl of sample buffer [22] and heated at 95 °C for 5 min.

In the experiments to study the secretory phosphoprotein, the parenchymal cells were challenged with non-parenchymal-liver-cell media or prostaglandins during 1 h. Subsequently the samples were rapidly cooled to 0 °C and centrifuged (1 min, 500 *g*), and the supernatant was centrifuged again (5 min at 20000 *g*); 50 μl of the resulting supernatant was mixed with 200 μl of sample buffer [22] and heated for 5 min at 95 °C.

The resulting samples were separated by one- [23] and two- [24] dimensional gel electrophoresis. Dried gels were exposed to Kodak SB-5 autoradiography films, and exposure times were checked to give a linear response [25]. Autoradiographs of one-dimensional gels were quantified with a Vitatron TLD 100 spectrophotometer. Spots from two-dimensional gels were cut out, and radioactivity was measured by liquid-scintillation counting.

Kupffer and endothelial liver cells were isolated by collagenase (type I) perfusion at 37 °C and subsequent counterflow centrifugation as described in [26], except for the first elutriation step, which was replaced by a centrifugation step (2 min, 75 *g*). Kupffer cells were > 90% pure; endothelial liver cells were 99% pure. The cells were incubated at 37 °C with constant shaking at 0.5–2 mg of protein/ml in RPMI 1640 medium, saturated with O_2/CO_2 (19:1), pH 7.4, keeping the viability > 95%. After a 10 min preincubation, the cells were washed and incubated again. After 1–2 h conditioned media were collected.

Phosphorylase *a* activity was determined in parenchymal-liver-cell extracts prepared as described in [13] with an assay described in [27] in the presence of 0.5 mM-caffeine.

RESULTS AND DISCUSSION

The influence of Kupffer- and endothelial-liver-cell media on the phosphorylation state of parenchymal-liver-cell proteins was studied. Fig. 1 shows that the phosphorylation of an M_r-63000 band is inhibited by both types of conditioned media, whereas the phosphorylation of an M_r-47000 and an M_r-97000 band is enhanced by both media. These data clearly show that media of both non-parenchymal-liver-cell types, i.e. Kupffer and endothelial cells, can influence the phosphorylation state of some specific parenchymal-cell proteins, whereas the phosphorylation state of most of the protein is unaffected. Since Kupffer cells can produce

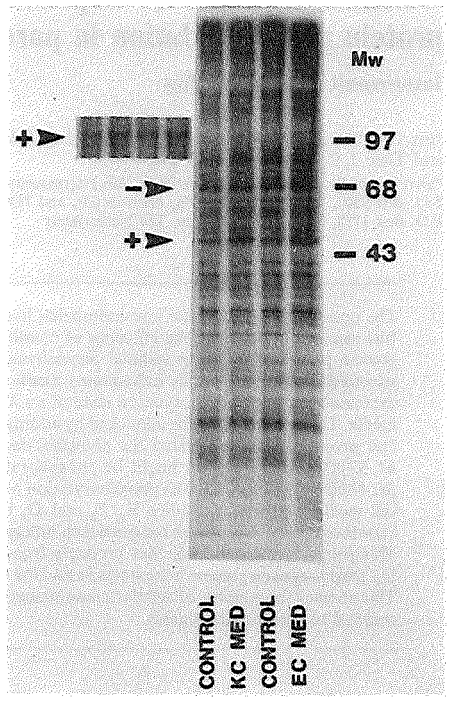

Fig. 1. Influence of conditioned Kupffer- (KC) and endothelial-liver-cell (EC) media on the phosphorylation state of parenchymal-liver-cell proteins

Parenchymal-cell suspension (150 μl) was challenged with 50 μl of conditioned media for 5 min. A longer-exposed film is also shown for the M_r-97000 band.

several prostaglandins [4–6], and endothelial liver cells were recently also shown to possess this capacity [28], we added prostaglandins in order to determine if prostaglandins could mimic the effect of non-parenchymal-liver-cell media.

As shown in Fig. 2 and Table 1, prostaglandins D_2 and E_2 have the same effect as the non-parenchymal-cell media on the phosphorylation state of parenchymal-cell proteins, i.e. inhibition of the phosphorylation of an M_r-63000 protein and enhanced phosphorylation of proteins of M_r 47000 and 97000. The facts that prostaglandins mimic the effect of Kupffer- and endothelial-liver-cell media, and are also produced by these cells [28], suggest that prostaglandins are the active factor in these media.

Since conditions media from Kuppfer and endothelial liver cells and prostaglandins bring about both enhanced and decreased phosphorylation of specific proteins in parenchymal liver cells, they are likely to act on specific intracellular targets which might involve both protein kinase and phosphatase activity. Further studies are, however, needed to establish the intracellular mechanism of action.

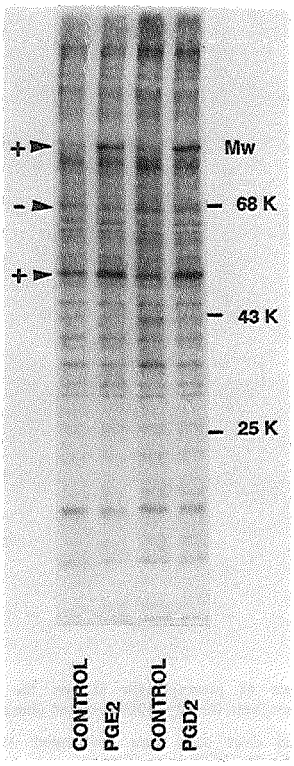

Fig. 2. Influence of prostaglandin (PG) D_2 and E_2 on the phosphorylation state of parenchymal liver cell proteins

Parenchymal liver cells were challenged with 1 μM-prostaglandins for 5 min.

It has been suggested [7–10] that in the regulation of glycogenolysis by phorbol ester and platelet-activating factor in parenchymal cells the effect is mediated by non-parenchymal cells. This suggestion is based on studies with perfused liver in which it is shown that the effect of phorbol ester and platelet-activating factor can be blocked by indomethacin [7–12,15,16]. More recently this hypothesis was substantiated in experiments showing that in the perfused liver phorbol ester stimulates production of prostaglandin D_2 [17]. Prostaglandin D_2 is the major eicosanoid product of both Kupffer and endothelial liver cells [28], and it stimulates glycogenolysis both in perfused liver and in isolated parenchymal liver cells [17]. Our present experiments with purified cells show that Kupffer and endothelial liver cells form prostaglandins in such quantities that effective specific changes in protein phosphorylation in parenchymal liver cells can be induced. The enzyme which regulates glycogenolysis in parenchymal liver cells is phosphorylase, and its activity depends completely on its phosphorylation state. It is likely that this enzyme

Table 1. Effect of conditioned Kupffer- and endothelial-liver-cell media and prostaglandins E_1, E_2 and D_2 on the phosphorylation state of the M_r-97000, M_r-68000 and M_r-47000 proteins

Data are expressed as means ± S.D. of four experiments (all values are significantly different from control, $P < 0.05$).

	Change in phosphorylation (fold over control)		
Protein...	M_r 97000	M_r 68000	M_r 47000
Kupffer-cell medium	2.7±0.4	0.62±0.04	2.3±0.3
Endothelial-cell medium	2.4±0.3	0.55±0.07	1.8±0.2
Prostaglandin E_1	5.8±0.7	0.61±0.04	1.9±0.3
Prostaglandin E_2	6.4±0.5	0.66±0.06	1.7±0.2
Prostaglandin D_2	9.3±0.6	0.74±0.05	1.8±0.1

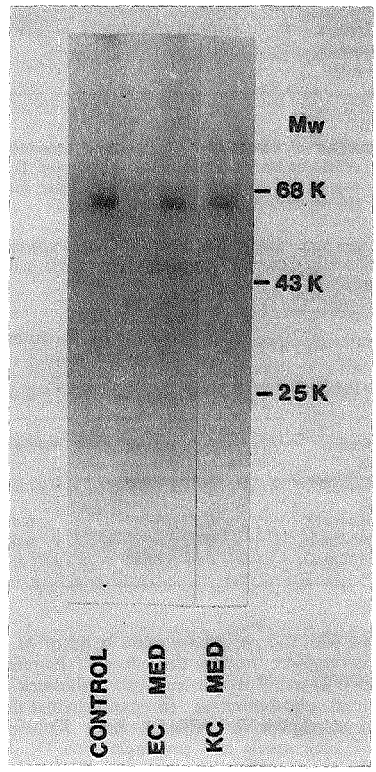

Fig. 3. Influence of conditioned Kupffer- (KC) and endothelial-liver-cell (EC) media on the amount of [^{32}P]phosphate in the M_r-63000 secreted phosphoprotein

Parenchymal-liver-cell suspension (350 μl) was challenged with 50 μl of conditioned media or 50 μl of RPMI medium (control) during 60 min.

Table 2. Effect of conditioned Kupffer- and endothelial-cell media and prostaglandins E_1, E_2 and D_2 on the activity of phosphorylase *a* in parenchymal-cell extracts

Results are expressed as means ± S.D. of four or five experiments; the level of significant difference from control is indicated. Parenchymal cells were challenged for 10 min with additives; 50 μl of medium was added to 450 μl of parenchymal-cell suspension.

	Phosphorylase *a* activity (mmol/h per mg of protein)	Stimulation factor
Control	31 ± 67	
Endothelial-cell medium	483 ± 67 ($P < 0.001$)	1.53
Kupffer-cell medium	633 ± 83 ($P < 0.005$)	2.00
Prostaglandin D_2 (1 mM)	550 ± 33 ($P < 0.001$)	1.74
Prostaglandin E_1 (1 μM)	517 ± 67 ($P < 0.001$)	1.63
Prostaglandin E_2 (1 μM)	483 ± 217 ($P < 0.05$)	1.53
Prostaglandins E_1, E_2 and D_2 (each 1 μM)	550 ± 83 ($P < 0.001$)	1.74

Table 3. Effect of conditioned Kupffer- and endothelial-liver-cellmedia and prostaglandins E_1, E_2 and D_2 on the amounts of [^{32}P]phosphate and [^{3}H]fucose detected in the M_r-63000 secreted phosphoprotein

Data are expressed as means ± S.D. of four experiments; *significantly different from control ($P < 0.05$); n.d., not determined.

	Change in incorporation (fold over control)	
	[^{32}P]Phosphate	[^{3}H]Fucose
Kupffer-cell medium	0.79 ± 0.04*	0.97 ± 0.03
Endothelial-cell medium	0.81 ± 0.05*	0.95 ± 0.04
Prostaglandin E_1	0.78 ± 0.05*	n.d.
Prostaglandin E_2	0.68 ± 0.97*	n.d.
Prostaglandin D_2	0.68 ± 0.06*	1.01 ± 0.02

correlates with the phosphorylation band of M_r 97000 [29]. We therefore studied the effect of Kupffer- and endothelial-liver-cell media and prostaglandins on the activity of phosphorylase in parenchymal-liver-cell extracts, as shown in Table 2. Both Kupffer- and endothelial-cell media and prostaglandins D_2, E_2 and E_1 stimulate phosphorylase activity. These data are consistent with the assumption that the M_r-97000 phosphoprotein is phosphorylase.

Parenchymal liver cells secrete a single phosphoprotein, of M_r 63000 and pI 5.0–5.5, which has been shown to be a negatively regulated acute-phase protein [18,19]. The influence of Kupffer- and endothelial-liver-cell media and prostaglandins D_2, E_2 and E_1 on the phosphorylation of this protein was studied. Fig. 3 shows that both Kupffer- and endothelial-cell media decrease the amount of [^{32}P]phosphate detected in the secreted phosphoprotein. The amount of [^{32}P]-phosphate was significantly decreased by about 20% (Table 3). The inhibitory influence of prostaglandin D_2 on the amount of [^{32}P]-phosphate detected in the secreted phosphoprotein is shown in Fig. 4. The amount of [^{32}P]phosphate in the secreted phosphoprotein was also inhibited by prostaglandins E_2 and E_2 (Table 3). To investigate the specificity of the decrease in [^{32}P]phosphate in the secretory phosphoprotein, experiments were performed in which the [^{3}H]fucose incorporation in the secreted M_r-63000 protein was quantified (previous data [18,19] showed a high content of glycoresidues. The incorporation of [^{3}H]fucose into the secreted phosphoprotein was not influenced by non-parenchymal-liver-cell media or prostaglandins, indicating that the phosphorylation was influenced selectively (Table 3).

In conclusion, the present data show that Kupffer and endothelial liver cells secrete products which can influence the phosphorylation state of some specific proteins in parenchymal liver cells. Since the same specific effects can be brought about by prostaglandins, it is likely that prostaglandins are the active components present in conditioned media of Kupffer and endothelial cells. Since

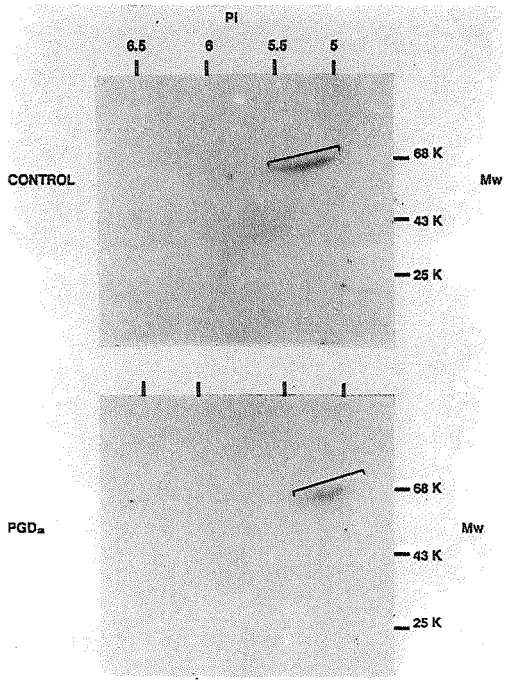

Fig. 4. Influence of prostaglandin D_2 on the amount of [^{32}P]phosphate the M_r-63000 secreted phosphoprotein

Parenchymal liver cells were challenged with 1 μM-prostaglandin D_2 (PGD_2) for 60 min.

prostaglandin D_2 is the most prominent prostaglandin produced by these cells, it is likely that the effects resulted from the action of prostaglandin D_2. These data sustain the concept of cellular communication between the various liver cell types, and it can be concluded that the products from non-parenchymal liver cells do not exert a general effect on parenchymal cells, but influence specific intracellular targets. These specific changes might form an additional possibility for the liver to adapt hepatic metabolism to extra-hepatic signals.

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APPENDIX PAPER IV

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Hormonal Control of Glycogenolysis in Parenchymal Liver Cells by Kupffer and Endothelial Liver Cells*

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Conditioned media of isolated Kupffer and endothelial liver cells were added to incubations of parenchymal liver cells, in order to test whether secretory products of Kupffer and endothelial liver cells could influence parenchymal liver cell metabolism. With Kupffer cell medium an average stimulation of glucose production by parenchymal liver cells of 140% was obtained, while endothelial liver cell medium stimulated with an average of 127%. The separation of the secretory products of Kupffer and endothelial liver cells in a low and a high molecular weight fraction indicated that the active factor(s) had a low molecular weight. Media, obtained from aspirin-pretreated Kupffer and endothelial liver cells, had no effect on the glucose production by parenchymal liver cells. Because aspirin blocks prostaglandin synthesis, it was tested if prostaglandins could be responsible for the effect of media on parenchymal liver cells. It was found that prostaglandin (PG) E_1, E_2, and D_2 all stimulated the glucose production by parenchymal liver cells, PGD_2 being the most potent. Kupffer and endothelial liver cell media as well as prostaglandins E_1, E_2, and D_2 stimulated the activity of phosphorylase, the regulatory enzyme in glycogenolysis. The data indicate that prostaglandins, present in media from Kupffer and endothelial liver cells, may stimulate glycogenolysis in parenchymal liver cells. This implies that products of Kupffer and endothelial liver cells may play a role in the regulation of glucose homeostasis by the liver.

The liver is a major site of glycogen storage and plays a crucial role in the homeostasis of blood glucose. Glycogen synthesis and breakdown are under strict hormonal regulation. Besides the well-known stimulators of glycogenolysis, *i.e.* glucagon and epinephrine, whose mode of action is well defined, other factors stimulate glycogenolysis. Recently, the effect of the tumor-promoting phorbol ester, phorbol-12-myristate 13-acetate (PMA)[1] on glucose release by the liver was studied, to test the possible involvement of protein kinase C (Ca^{2+}/phospholipid-dependent enzyme) in the regulation of glycogenolysis. PMA stimulated glycogenolysis in the perfused liver (1) but failed to stimulate in isolated parenchymal liver cells, the cellular site of glycogen storage (6–8). With platelet-activating factor (PAF), similar observations have been reported (2–5) although the mechanism of action of platelet-activating factor might not involve activation of protein kinase C.

Besides parenchymal cells other cell types are present in the liver, *i.e.* Kupffer cells, endothelial liver cells, fat-storing cells, and pit cells (9). Since PMA and PAF both act on intact liver but fail to affect glycogenolysis in isolated parenchymal liver cells, the possibility was raised that non-parenchymal liver cells may mediate the regulatory effects of PMA and PAF on glycogenolysis in the liver. The present study was undertaken to test whether Kupffer or endothelial liver cells secrete factors that may influence parenchymal liver cell metabolism.

In recent years, techniques have been developed which enable the isolation and purification of parenchymal liver cells, Kupffer cells, and endothelial liver cells (10). The isolated cells have been used to study, *e.g.* the relative importance of the different cell types in the receptor mediated uptake of lipoproteins (10–12). These isolation techniques were applied to assess the relationship between various cell types in the regulation of glycogenolysis in the liver.

MATERIALS AND METHODS

Prostaglandins and collagenase type I and IV were from Sigma, *O*-acetylsalicylic acid (aspirin) was from BDH, and other chemicals were of Pro Analyse quality.

Male Wistar rats, fed *ad libitum*, weighing 200–220 g were used. 18 mg of nembutal was given intraperitoneally for anesthesia, usually performed between 9.00–10.00 a.m.

Parenchymal liver cells were isolated after 20 min of collagenase (type IV, 0.1%) perfusion by the method of Seglen (13), modified as previously described (14). Parenchymal liver cells were incubated at 37 °C under constant shaking at 5 mg of protein/ml in Krebs-Ringer bicarbonate buffer, saturated with O_2/CO_2 (95%:5%), pH 7.4, keeping the viability of the cells >95%. At 10-min intervals aliquots of cell suspensions were withdrawn, rapidly cooled to 0 °C, centrifuged at 500 × *g* for 5 min, and subsequently glucose was determined in the supernatant by the glucose oxidase-ABTS method (15): Zero time values were determined as follows: aliquots of the cell suspension were withdrawn and after cooling, stimuli (conditioned media or prostaglandins) were added and samples were prepared for glucose determination similar as with the other time points.

Kupffer and endothelial liver cells were isolated by collagenase (type I) perfusion at 37 °C. After collagenase digestion the liver was excised, cut into pieces, and filtered through a nylon gauze. Parenchymal and non-parenchymal cells were separated by differential centrifugation. In a subsequent centrifugation on a metrizamide gradient, cell debris was removed from the non-parenchymal cell fraction. Finally, Kupffer and endothelial liver cells were purified by counterflow centrifugation in a Beckman elutriation rotor. The method has been described in detail elsewhere (10) and was employed

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¶ To whom correspondence should be addressed.

[1] The abbreviations used are: PMA, phorbol-12-myristate 13-acetate; PAF, platelet-activating factor.

here with the exception of the first elutriation step which was replaced by a centrifugation step (2 min at 75 × *g*). Kupffer cells were >90% pure, endothelial liver cells were >95% pure. A differentiation between Kupffer and endothelial liver cells was made by peroxidase staining and Papanicolau counterstaining (16, 17). The cells were incubated at 37 °C under constant shaking at 0.5–2 mg of protein/ml in RPMI 1640 (glucose free) saturated with O_2/CO_2 (95%:5%) at pH 7.4, keeping the viability >95%. After a 10-min preincubation, the cells were washed and incubated again. After 1 or 2 h, conditioned media were collected by centrifugation for 5 min at 500 × *g*, subsequent supernatants were again centrifuged for 10 min at 10,000 × *g*. Prostanoid-free media were obtained by preincubating Kupffer and endothelial liver cells in the presence of 2 mM *O*-acetylsalicylic acid for 1 h, after which the cells were washed (to remove aspirin) and incubated for 1 h, and subsequently the conditioned media were collected as described above.

Phosphorylase *a* activity was determined in parenchymal liver cell extracts prepared by freezing and thawing as described by Vander Werve *et al.* (7), with an assay described by Stalmans and Hers (18). This assay was performed in the presence of 0.5 mM caffeine, to fully depress phosphorylase *b*.

Low molecular weight components were removed from media by centrifuging them through Sephadex G-25 by the method of Penefsky (19).

Data were statistically analyzed with a 1-tailed, paired Student's *t* test.

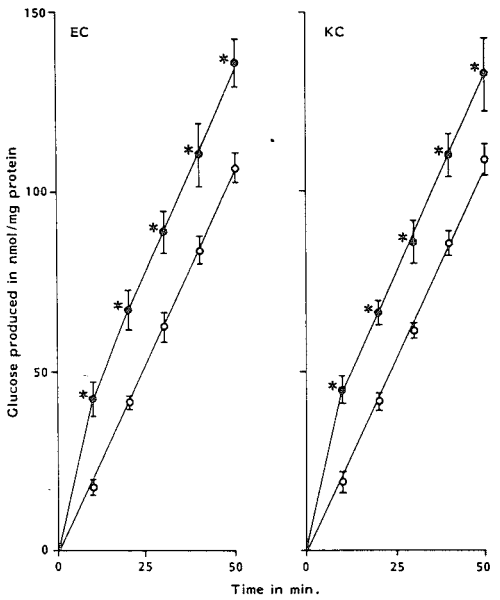

FIG. 1. **Effect of conditioned media of Kupffer (*KC*) and endothelial liver cells (*EC*) on the amount of glucose produced by isolated parenchymal cells.** 50 μl of conditioned medium of Kupffer or endothelial liver cells were added to 450 μl of a parenchymal cell suspension (●) and compared to a control incubation (○). For each incubation zero time values were determined as described under "Materials and Methods." Data are mean ± S.E. of five experiments; * indicates significant difference from control (at 10 and 20 min, $p < 0.005$; at other time points, $p < 0.05$).

RESULTS

In Fig. 1 it is shown that conditioned media of Kupffer and endothelial liver cells stimulate the glucose production of isolated parenchymal liver cells. For endothelial liver cell media, the increase in glucose production after 10 min was 127 ± 44%, for Kupffer cell media an increase of 140 ± 59% was found. The zero time values for the assay performed in the presence of endothelial- or Kupffer-conditioned medium were not different from the control, thus indicating that no glucose was present or formed in these media. The time course of the percentual stimulation by Kupffer and endothelial liver cell media is shown in Fig. 2. The increase in glucose production is maximal at 10 min after addition of media and then the stimulation declines. A similar time course is seen for the effect of glucagon on the glucose production by parenchymal liver cells. The maximal effect of glucagon is about twice as high as the effect of the conditioned media.

In order to investigate the nature of the stimulatory factor(s) present in conditioned media of Kupffer and endothelial liver cells, low molecular weight components of the conditioned media were removed by gel filtration on Sephadex G-25. After removal of low molecular weight components, the stimulatory effect of endothelial liver cell and Kupffer cell media was mostly abolished (Fig. 3). These data suggest the involvement of low molecular weight factors in the stimulatory effect of non-parenchymal liver cell media on the glucose production by parenchymal liver cells. Since prostaglandins may act as intercellular messengers, the effect of individual prostaglandins on the glucose production by isolated parenchymal liver cells was studied. It appeared that prostaglandin E_1, E_2, and D_2 stimulate the glucose production by isolated parenchymal liver cells (Fig. 4). The stimulation at 10 min

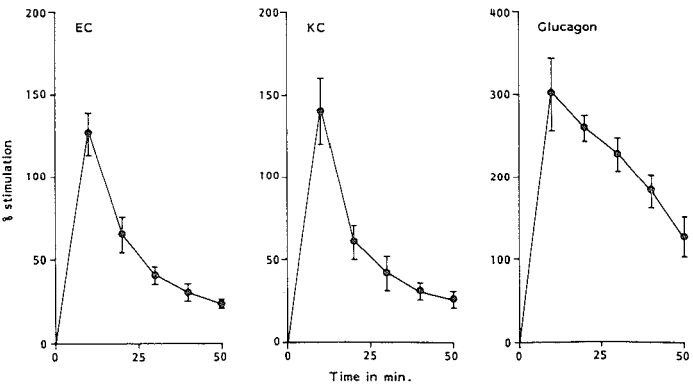

FIG. 2. **Time course of the percentual stimulation of glucose produced by parenchymal liver cells, by addition of Kupffer (*KC*) and endothelial liver cell- (*EC*) conditioned media and glucagon (10^{-7} M).** 50 μl of media were added to 450 μl of parenchymal cell suspension. Data are mean ± S.E. of seven experiments.

after addition of prostaglandin E_1 (21 ± 4%, $n = 4$) and prostaglandin E_2 (28 ± 14, $n = 4$) was significantly smaller than the stimulation by prostaglandin D_2 (68 ± 12%, $n = 5$) and a mixture of prostaglandins E_1, E_2, and D_2 (63 ± 11%, $n = 4$).

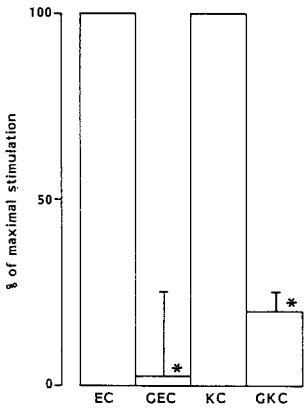

FIG. 3. **Effect of gel filtration of Kupffer (*KC*) and endothelial cell- (*EC*) conditioned media on their stimulation of glucose production by parenchymal liver cells.** 50 μl of untreated (*EC, KC*) or gel filtrated (*GEC, GKC*), *i.e.* low molecular weight components-depleted, media were added to 450 μl of parenchymal cell suspension and compared to incubations without gel filtration (*EC, KC*). Data are means ± S.D. of four experiments; * indicates significant difference from control (*EK, KC*) ($p < 0.005$).

The time course of stimulation of glucose production by parenchymal liver cells by prostaglandin D_2 was similar to that by Kupffer and endothelial liver cell media (Fig. 5). Prostaglandins E_1 and E_2 also had a similar time course of stimulation (data not shown).

Since it is known that Kupffer cells can produce several prostaglandins (20–22), it seems possible that prostaglandins are the active factor(s) in the conditioned media of Kupffer and endothelial liver cells. To test this hypothesis, Kupffer and endothelial liver cells were preincubated in the presence of aspirin, a well-known irreversible inhibitor of cyclooxygenase, to obtain prostanoid-free conditioned media. These were obtained from cells preincubated for 1 h with aspirin, the cells were washed in order to remove aspirin and subsequently incubated for 1 h in order to obtain the conditioned media.

Conditioned media of endothelial liver cells gave a stimulation at 10 min of 208 ± 61% ($n = 4$), aspirin preincubation reduced the stimulation by endothelial liver cell media completely. The stimulation at 10 min by conditioned Kupffer cell media of 149 ± 39% was also reduced completely by pretreatment of Kupffer cells with aspirin (Fig. 6).

In order to verify the intracellular target in parenchymal liver cells of Kupffer and endothelial cell media and prostaglandins, we measured the activity of phosphorylase, the enzyme responsible for glycogen breakdown and considered to be the regulatory site for this process.

In Table I it is shown that at 10 min after addition of Kupffer or endothelial cell media or prostaglandin E_1, E_2, or D_2, the phosphorylase activity, measured in parenchymal liver cell extracts, is stimulated. If measured 30 min after addition, this stimulation had disappeared (data not shown), resembling the time course shown in Figs. 2 and 5 for glucose production.

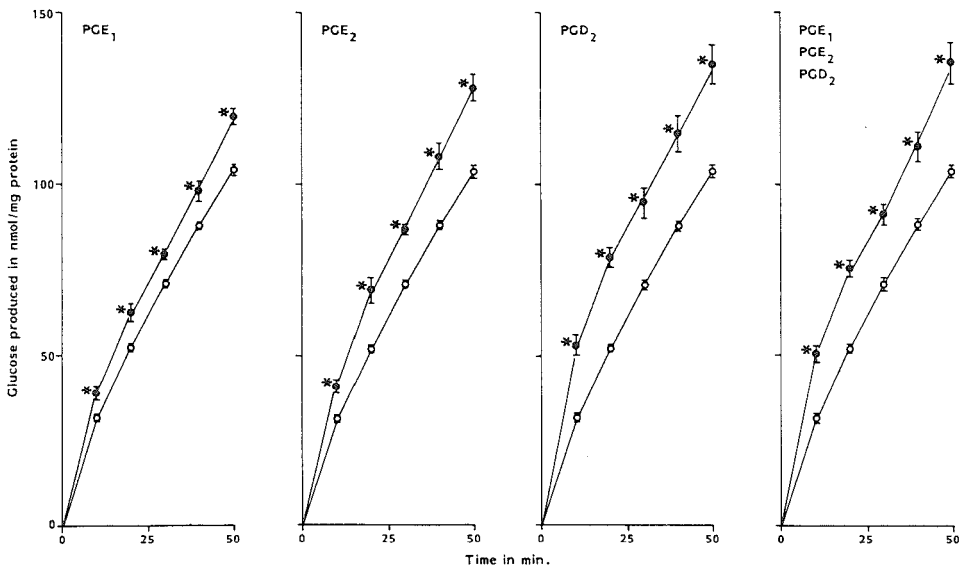

FIG. 4. **Effect of prostaglandins E_1, E_2, and D_2 on the glucose production by parenchymal liver cells.** Prostaglandins were added in a 10^{-6} M concentration (●) and compared to control incubations (○). For each addition zero time values were determined as discussed under "Materials and Methods." Data are mean ± S.E. of four experiments; * indicates significant difference from control ($p < 0.05$ for PGE_1 and PGE_2, $P < 0.002$ for PGD_2 and mixture).

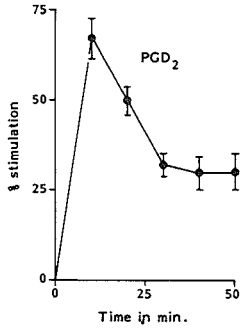

FIG. 5. **Time course of prostaglandin D_2 stimulation of glucose production by parenchymal liver cells.** Data are mean ± S.E. of four experiments.

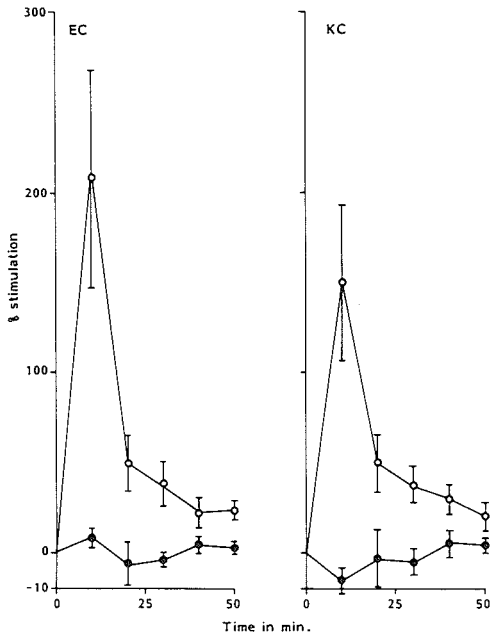

FIG. 6. **Effect of aspirin preincubation of Kupffer (*KC*) and endothelial (*EC*) liver cells on the stimulatory effect of Kupffer and endothelial cell media on the glucose production by parenchymal cells.** 50 μl conditioned medium of untreated (○) or aspirin-preincubated (●) Kupffer or endothelial liver cells were added to 450 μl of a parenchymal liver cell suspension and compared to control incubations. Zero time values were determined as described under "Materials and Methods." Data are expressed as mean stimulation (%) ± S.E. of four experiments.

TABLE I

Effect of Kupffer- and endothelial cell-conditioned media and prostaglandins E_1, E_2, and D_2 on the activity of phosphorylase a in parenchymal cell extracts

Results are expressed as means ± S.D. of 4–5 experiments, and the level of significant difference from control is indicated. Parenchymal cells were challenged for 10 min with additives. 50 μl of media was added to 450 μl of parenchymal cell suspension.

	Phosphorylase activity	
	nmol glucose produced/mg protein/min	% stimulation
Control	19 ± 4	
Endothelial cell medium	29 ± 4 ($p < 0.001$)	55
Kupffer cell medium	38 ± 5 ($p < 0.005$)	97
Prostaglandin E_1, 10^{-6} M	31 ± 4 ($p < 0.001$)	60
Prostaglandin E_2, 10^{-6} M	29 ± 13 ($p < 0.05$)	55
Prostaglandin D_2, 10^{-6} M	33 ± 2 ($p < 0.001$)	71
Prostaglandin E_1, E_2, D_2, 10^{-6} M	33 ± 5 ($p < 0.001$)	71

DISCUSSION

Products of Kupffer and endothelial liver cells were shown to enhance the glucose production of isolated parenchymal liver cells. Because of limiting experimental conditions, *e.g.* the yield of Kupffer and endothelial liver cells obtained during isolation, it was not possible to exactly define the maximal extent of stimulation. With different amounts of medium of the same batch of cells, we achieved a near-linear dose response relationship (data not shown), indicating that maximal stimulation had not yet been reached.

Non-parenchymal liver cells synthesize and secrete several products including various proteins (27, 28). Gel filtration indicated that the factor(s) in non-parenchymal liver cell media responsible for the effect on glucose production by parenchymal liver cells, was (were) not of high molecular weight nature. In the low molecular weight region, prostaglandins E_1 and E_2 were shown earlier to be important products of Kupffer cells (20–22). Results from our laboratory (30) show that endothelial liver cells also produce several prostaglandins and that the main prostaglandin present in conditioned media of both Kupffer and endothelial liver cells is prostaglandin D_2. For this reason we further explored the possibility that prostaglandins are the stimulating factor(s) in non-parenchymal cell media.

Prostaglandin D_2 added to parenchymal liver cells proved to be the most effective prostaglandin in stimulating glucose production and since it is the most abundant eicosanoid product of both Kupffer and endothelial liver cells (30), it seems to be a good candidate for the putative factor(s) in non-parenchymal cell media. The stimulation obtained by high concentrations (10^{-6} M) of prostaglandins should be expected to be larger than that of non-parenchymal liver cell media. It seems possible, however, that a physiological combination of prostanoids is needed for the full expression of their effect. Furthermore, it should be realized that prostaglandins are rapidly metabolized by parenchymal liver cells (23–26), resulting in a decline in prostaglandin concentration during the incubation. The prostanoid nature of the non-parenchymal liver cell media factor(s) which is (are) responsible for the stimulation of glucose production in parenchymal liver cells is also strongly suggested by the finding that preincubation of Kupffer and endothelial liver cells with aspirin fully depresses the stimulatory effect of non-parenchymal liver cell media. Since aspirin is an irreversible inhibitor of cyclooxygenase, it blocks the formation of prostaglandins.

The intracellular target, which mediates the increase in glucose production, is shown to be phosphorylase, and its activity was found to be increased under conditions stimulatory for glucose production.

The possible involvement of factors of non-parenchymal liver cell types in the regulation of glucogenolysis has been suggested on the basis of experiments with the perfused liver (1–8). PMA and PAF were shown to stimulate glycogenolysis in the perfused liver but failed to act on isolated parenchymal liver cells. Furthermore, the glycogenolytic effect of PMA and

PAF in perfused liver can be abolished by the cyclooxygenase inhibitor indomethacin and the phospholipase A_2 inhibitor bromophenacyl-bromide (1, 4, 29), suggesting that prostanoids may mediate the PMA and PAF stimulation of glycogenolysis. Our experiments with the reconstituted liver cell system directly prove that both Kupffer and endothelial liver cells can secrete factors, probably prostaglandins, that can modulate parenchymal cell metabolism. The physiological involvement of the non-parenchymal liver cell types in the regulation of the glucose homeostasis maintained by parenchymal liver cells clearly extends the regulatory potential of this process and introduces the concept of cellular communication as an additional system for metabolic regulation in the liver.

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APPENDIX PAPER V

Biochem. J. (1988) 250, 77–80 (Printed in Great Britain)

Prostaglandin D_2 mediates the stimulation of glycogenolysis in the liver by phorbol ester

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The tumour-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), when added to the perfused liver, stimulates glycogenolysis 2-fold. This stimulation is not seen when aspirin is present in the perfusion medium. In isolated parenchymal liver cells, PMA is not able to stimulate glycogenolysis, suggesting that its effect on glycogenolysis might be indirect and depends on the presence of the non-parenchymal liver cell types. To test the possible operation of an indirect mechanism, we measured the amount of prostaglandin (PG) D_2 in liver perfusates. After addition of PMA, the amount of PGD_2 is doubled, in parallel with the increase in glycogenolysis. Glycogenolysis in both isolated parenchymal liver cells and perfused liver could be stimulated by the addition of PGD_2. Our data indicate that stimulation of glycogenolysis in the liver by PMA may be mediated by non-parenchymal liver cells, which produce PGD_2 in response to PMA. Subsequently PGD_2 activates glycogenolysis in the parenchymal liver cells. The intercellular communication inside the liver in response to PMA adds a new mechanism to the complex regulation of glucose homoeostasis by the liver.

INTRODUCTION

Parenchymal liver cells are the site of hepatic glycogen storage. The synthesis and breakdown of glycogen in these cells is under strict hormonal regulation. Glycogenolysis is regulated at the site of phosphorylase. Glucagon activates phosphorylase through a cyclic-AMP-dependent mechanism [1,2]. Ca^{2+}-linked hormones such as angiotensin II, vasopressin and α_1-adrenergic agents also stimulate phosphorylase activity [1,2]. These hormones act via two different second messengers, i.e. inositol 1,4,5-trisphosphate, which triggers Ca^{2+} mobilization [3,4], and 1,2-diacylglycerol, which activates protein kinase C [5,6].

Raising the intracellular Ca^{2+} concentration by the addition of Ca^{2+} ionophore A23187 to isolated parenchymal liver cells results in increased phosphorylase activity [7]. Surprisingly, the phorbol ester PMA, a potent activator of protein kinase C, does not increase the phosphorylase activity in isolated parenchymal liver cells [8], or only very weakly [9] or at very high concentration [10]. In perfused liver [11–13], however, PMA does stimulate glycogenolysis, suggesting that in the intact liver the presence of liver cell types other than parenchymal cells may be involved in the stimulation of glycogenolysis by PMA. Platelet-activating factor has the same effect on glycogenolysis as PMA; it stimulates glycogenolysis in perfused liver, but fails to do so in isolated parenchymal liver cells [14–16]. Since the stimulation of glycogenolysis in perfused liver by PMA and platelet-activating factor is blocked by indomethacin, the involvement of prostaglandins has been suggested [12,17]. Patel [13] suggested that PMA causes vasoconstriction, which results in hypoxia, which in its turn triggers glycogenolysis in the parenchymal cells.

The liver contains, in addition to parenchymal cells, Kupffer cells, endothelial cells, fat-storing cells and pit cells [18]. Kupffer cells are known to produce several prostaglandins [19–21]. Recently it was shown [22] that endothelial liver cells also produce several prostaglandins. Evidence was obtained showing that the major prostaglandin produced by both Kupffer and endothelial liver cells is prostaglandin (PG) D_2 [22].

In the present work we investigated the possible role of PGD_2 as a mediator of the glycogenolytic effect of PMA in the intact liver.

MATERIALS AND METHODS

PMA, PGD_2 and collagenase type IV were from Sigma; *O*-acetylsalicylic acid (aspirin) was from BDH; PGD_2 radioimmunoassay kit was from Amersham; glucagon was from Novo.

Male Wistar rats, fed *ad libitum*, weighing 200–220 g were used. Nembutal (18 mg) was given intraperitoneally for anaesthesia, usually performed between 9:00 and 10:00 h.

Parenchymal liver cells were isolated after perfusion for 20 min with collagenase (type IV; 0.1%) by the method of Seglen [23], modified as previously described [24]. Parenchymal liver cells were incubated at 37 °C under constant shaking at 5 mg of protein/ml in Krebs–Ringer bicarbonate buffer (1.3 mM-$CaCl_2$), saturated with O_2/CO_2 (19:1), pH 7.4, which keeps the viability of the cells > 95%. At 10 min intervals, portions of cell suspension were withdrawn, rapidly cooled to 0 °C, centrifuged at 500 *g* for 5 min, and subsequently glucose was determined in the supernatant by the glucose oxidase–ABTS method [25].

For liver-perfusion experiments, the portal vein was

Abbreviations used: PMA, phorbol 12-myristate 13-acetate; PG, prostaglandin.

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cannulated and the liver was perfused with non-recirculating Krebs–Ringer bicarbonate buffer [23]. The $CaCl_2$ concentration was 1.3 mM. The perfusion buffer was kept saturated with O_2/CO_2 (19:1) and the pH was 7.4. The perfusion flow was 34 ml/min, and the temperature was kept at 37 °C. Before additions were tested, the liver was pre-perfused for 40–50 min to obtain a constant glucose output. At 1 min intervals effluent was collected, in which glucose was determined by the glucose oxidase–ABTS method [25].

PGD_2 was determined in the effluent with a PGD_2 radioimmunoassay kit from Amersham. The zero value [26] was determined in the effluent of aspirin-treated (2 mM) livers and subtracted from other values to correct for non-specific binding.

Data were statistically analysed with a one-tailed paired Student's *t* test.

RESULTS

In the perfused liver the influence of PMA on the glucose output was studied. Fig. 1 shows that PMA stimulates glucose output; this stimulation has a lag phase of 3–6 min and consists in all experiments of two peaks. The stimulation by PMA (60 ng/ml) is almost 2-fold, and glucagon at 0.1 μM gives an almost 4-fold stimulation. When the perfusion is performed in the presence of 2 mM-aspirin, PMA stimulation is abolished, whereas the stimulation by glucagon is unaffected.

When PMA (60 ng/ml) was added to isolated parenchymal liver cells (Fig. 2), no stimulation of glucose production occurred, although 0.1 μM-glucagon gave a 4-fold stimulation.

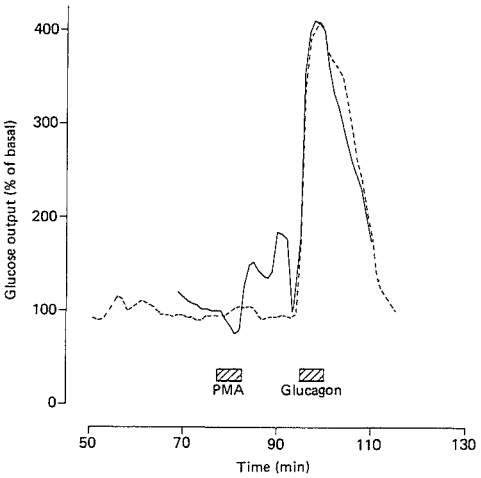

Fig. 1. Effect of PMA, glucagon and aspirin on the glucose output of perfused liver

Glucose was determined at 1 min intervals. PMA (60 ng/ml) and glucagon (0.1 μM) were given in 5 min pulses. In separate experiments livers were perfused with Krebs–Ringer buffer with (------) or without (——) aspirin present (2 mM). Data are from one typical experiment of six.

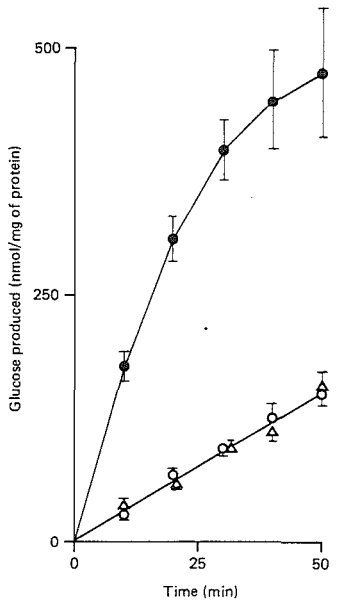

Fig. 2. Effect of PMA and glucagon on the glucose production by isolated parenchymal liver cells

○, Control; △, PMA (60 ng/ml); ●, glucagon (0.1 μM). Data are means ± S.E.M. for four experiments.

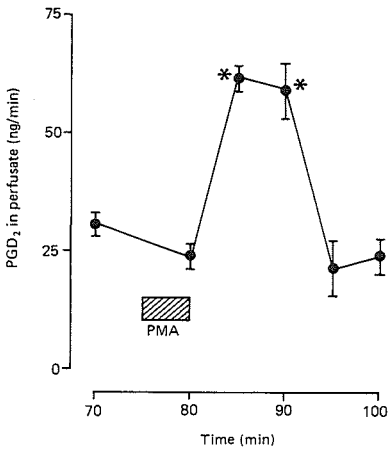

Fig. 3. Effect of PMA on PGD_2, recovered in liver perfusates

PMA (60 ng/ml) was given in a 5 min pulse. Data are means ± S.E.M. for four experiments; * indicates significant difference from control ($P < 0.05$).

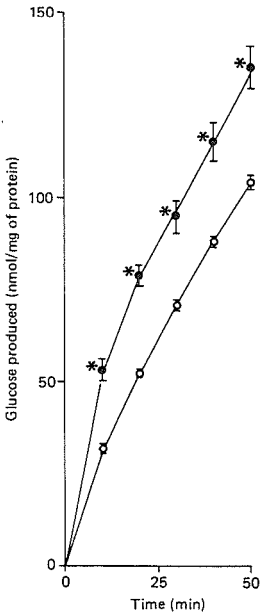

Fig. 4. Effect of PGD_2 on the glucose production by isolated parenchymal liver cells

○, Control; ●, PGD_2 (1 μM). Data are means ± S.E.M. for four experiments; * indicates significant difference from control ($P < 0.05$).

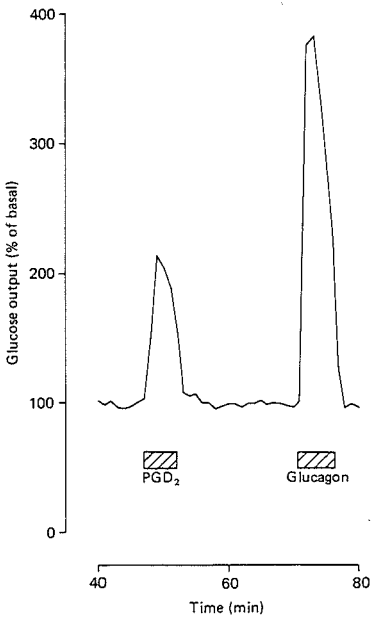

Fig. 5. Effect of PGD_2 and glucagon on the glucose output of perfused liver

Glucose was determined at 1 min intervals. PGD_2 (1 μM) and glucagon (0.1 μM) were given in 5 min pulses. Data are from one typical experiment of three.

In perfusates obtained in experiments as described in Fig. 1, PGD_2 was determined in a radioimmunoassay. As shown in Fig. 3, PMA (60 ng/ml) temporarily stimulates PGD_2 production by the liver. As with the stimulation of glucose production, there is a lag phase of approx. 5 min. PGD_2 recovered in the perfusate is more than doubled after addition of PMA. At 15 min after PMA stimulation, PGD_2 returns at the starting value; subsequent glucagon infusion did not affect the PGD_2 production (results not shown).

The influence of PGD_2 on the glucose production by isolated parenchymal liver cells was investigated (Fig. 4). PGD_2 at 1 μM stimulates glucose production in isolated parenchymal liver cells; the percentage stimulation is largest (70 %) at 10 min after addition.

In perfused liver, PGD_2 at 1 μM gives a more than 2-fold increase in glucose output (Fig. 5). The glucose output rises immediately after the addition of PGD_2 and begins to decline before the PGD_2 pulse has ended. The stimulation of glycogenolysis by PGD_2 was not affected by aspirin (cf. Fig. 1).

DISCUSSION

Our results confirm that PMA can stimulate glycogenolysis in perfused liver [11,12]. In agreement with reference [12], we observed a biphasic stimulatory response.

Since PMA does not stimulate glycogenolysis in isolated parenchymal liver cells (see also [8]), the intact liver or the presence of other liver cell types seems to be needed for a glycogenolytic response. The stimulation of glycogenolysis by PMA in the perfused liver was found to be abolished by including aspirin in the perfusion medium. Also, another inhibitor of prostanoid synthesis, indomethacin, abolishes the stimulation of PMA [12]. Therefore it seems likely that the stimulation of glycogenolysis by PMA is transduced by prostanoids. For Kupffer and endothelial liver cells, it is known that they can produce prostaglandins [19–22], and recently we obtained evidence that their major eicosanoid product is PGD_2 [22]. We therefore monitored the response of PGD_2 in the perfusate, as a consequence of PMA addition, and found that PGD_2 recovered in the perfusate is more than doubled on addition of PMA, indicating an increased production of PGD_2 by the liver. Although both Kupffer and endothelial liver cells can produce PGD_2, the capacity of Kupffer cells is much higher, so probably this cell type gives a major contribution.

The rise in PGD_2 occurs after a 5 min lag phase, which is also noticed in the glucose output. Because infusion of PGD_2 leads to an immediate increase in glucose output, it appears that the time-dependence of glucose output is consistent with an intermediate role of PGD_2. Thus our data suggest a causal relationship between increased PGD_2 production and stimulation of glucose production by PMA. Such a mechanism is consistent with recent observations in peritoneal macrophages that protein

kinase C is involved in the activation of eicosanoid synthesis [27]. The stimulation of glucose production in isolated parenchymal liver cells by PGD_2 shows that prostaglandins can act directly on these cells as glycogenolytic agents. For PMA and platelet-activity factor, and also for heat-aggregated IgG, stimulation of glycogenolysis in the liver via an indirect mechanism has been reported [28]. In that study, indomethacin was shown to block the stimulation of glycogenolysis in perfused liver by heat-aggregated IgG, and infusion of PGE_2 led to increased glycogenolysis. It was suggested that PGE_2 stimulated glycogenolysis via an induction of hepatic vasoconstriction, a mechanism that has also been suggested for platelet-activating-factor-stimulated glycogenolysis in perfused liver [29]. Here we show that PGD_2 has a stimulatory effect on glycogenolysis in both perfused liver and isolated parenchymal liver cells. Furthermore, we show here that PGD_2 production in the liver is stimulated by PMA, whereas production of PGE_2 and 6-oxo-$PGF_{1\alpha}$ has been reported not to be affected by PMA [13].

Considering the fact that PGD_2 is the most prominent prostaglandin produced by both Kupffer and endothelial liver cells [22], our data indicate that PGD_2 produced in non-parenchymal liver cells in response to PMA can directly stimulate glycogenolysis in parenchymal liver cells. Our data, however, do not exclude the possibility that, besides a direct effect of PGD_2 on glycogenolysis in parenchymal liver cells, an indirect effect of PGD_2 on glycogenolysis via vasoconstriction leading to hypoxia may occur.

The finding that products of non-parenchymal liver cell types may mediate the response to PMA adds a new type of mechanism to the complex regulation of glucose homoeostasis by the liver, and may also be relevant under pathophysiological conditions.

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APPENDIX PAPER VI

ENDOTOXIN STIMULATES GLYCOGENOLYSIS IN THE LIVER BY MEANS OF INTERCELLULAR COMMUNICATION

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SUMMARY

E.coli endotoxin (lipopolysaccharide) was shown to increase glycogenolysis in the perfused liver 2-3 fold. In isolated parenchymal liver cells, however, endotoxin did not influence glycogenolysis, whereas stimulation by endotoxin of glycogenolysis in the perfused liver could be blocked by aspirin. This suggests that the effect of endotoxin on liver glycogenolysis is mediated by eicosanoids. The amount of prostaglandin D_2 (which is the major prostanoid formed by Kupffer cells) in the liver perfusates was increased 5-fold upon endotoxin addition with a time course which preceeded the increase in glucose output.

It is concluded that endotoxin stimulates glycogenolysis in the liver by stimulating prostaglandin D_2 release from Kupffer cells, with a subsequent activation of glycogenolysis in parenchymal liver cells. This mechanism of intercellular communication may be designed to provide the carbohydrate source of energy necessary for the effective destruction of invaded microorganisms, by phagocytic cells, including the Kupffer cells.

INTRODUCTION

Gram negative septic shock is thought to be caused by endotoxins (lipopolysaccharides) derived from the cell wall of gram-negative bacteria (1-3). One of the most prominent effects of sepsis is the impairment of glucose homeostasis (4), caused by alterations in glycogen metabolism. Early or mild endotoxemia generates hyperglycemia, followed by hypoglycemia in prolonged endotoxemia (4, 5).

In the perfused liver heat aggregated immunoglobulin (HAG) which is like endotoxin (5, 6) thought to be taken up in the liver by Kupffer cells, was shown to stimulate glycogenolysis (7). HAG, however, could not stimulate glycogenolysis in isolated parenchymal liver cells indicating that Kupffer cells might mediate its glycogenolytic effect in the perfused liver. Since the glycogenolytic response to HAG in the perfused liver could be blocked by indomethacin, prostanoids were suggested to mediate this effect. Recently we reported that isolated Kupffer and endothelial liver cells secrete low molecular weight factors, presumably prostaglandins, which stimulate glucose production by parenchymal cells (8). The major prostanoid produced by the non-parenchymal cell types is prostaglandin D_2 (9) and therefore in the present work we focussed our attention upon the possibility that prostaglandin D_2 may mediate the effect of endotoxin on glucose metabolism inside the liver.

MATERIALS AND METHODS

The portal vein of anesthetized male Wistar rats, fed ad libitum, weighing 200-220 g, was cannulated and the liver was perfused with non-recirculating Krebs-Ringer buffer (10) at 34 ml/min. The perfusion buffer was kept saturated with O_2/CO_2 (95%/5%) at pH 7.4 and 37°C. Before endotoxin (LPS, Sigma, 026/B6) was added, the liver was preperfused for 50 min in the absence or presence of 2 mM aspirin (LHD). LPS (100 µg/ml) was given as a 5 min pulse. At 1 min intervals, effluent was collected and glucose was determined with the glucose-oxidase ABTS method (11).

Parenchymal liver cells were isolated by the method of Seglen (12) and modified as previously described (13). The cells were kept at 37°C, under constant shaking, at 5 mg protein/ml in Krebs-Ringer bicarbonate buffer, saturated with O_2/CO_2 (95%/5%) pH 7.4. Viability of the cells was more than 95%. At 10 min intervals aliquots of cell suspension were withdrawn, rapidly cooled to 0°C, centrifuged at 500 x g for 5 min and subsequently glucose was determined in the supernatant by the glucose-oxidase ABTS method (11).

Prostaglandin D_2 was determined with a specific radioimmunoassay (Amersham). Zero level was determined in effluent of aspirin treated livers and subtracted in order to correct for non-specific binding.

RESULTS AND DISCUSSION

E.Coli endotoxin (lipopolysaccharide, LPS) raised the glucose output of the perfused liver to 2.5 times the basal rate (Fig. 1). This increase in glucose production reflects glycogenolysis rather than gluconeogenesis since livers of fed animals were used and no exogenous gluconeogenic substrates were added. Morever, gluconeogenesis from endogenous substrates has been shown to be unaffected by endotoxin whereas gluconeogenesis from various exogenous substrates is impaired (22). The present finding is in agreement with the fact that in rat liver the glycogen content is decreased by 85% and the phosphorylase activity is increased by 35-40% at 4 hrs after endotoxin injection (14). Parenchymal liver cells, which form the site of hepatic glycogen storage, could however not be stimulated directly by endotoxin. When endotoxin

was added to freshly isolated parenchymal liver cells, glucose production by these cells was not influenced (Fig. 2). This finding suggests that the effect of endotoxin on glucose output by the liver may be mediated by non-parenchymal liver cells. The non-parenchymal liver cell types can be subdivided into Kupffer cells, endothelial cells, fat storing cells and pit cells (15). Of these cell types specifically the Kupffer cells (which are the liver macrophages) were demonstrated to take up radiolabeled endotoxin (6).

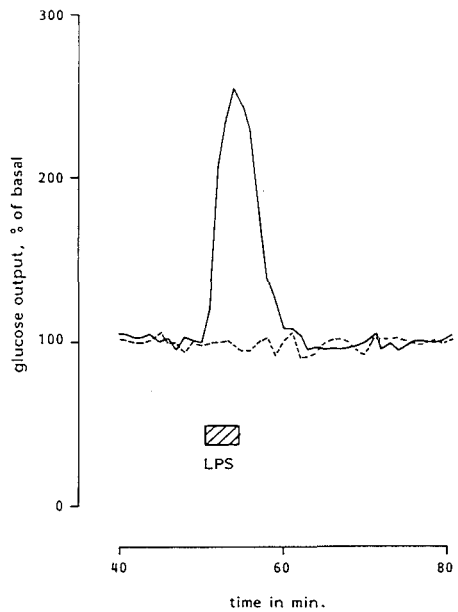

Figure 1. **Influence of endotoxin on the glucose output of perfused liver in the absence or presence of aspirin.**

The liver was preperfused for 50 min in the absence (solid line) or presence of 2 mM aspirin (broken line). LPS (100 µg/ml) was given as a 5 min pulse. Data are of a typical experiment out of 3.

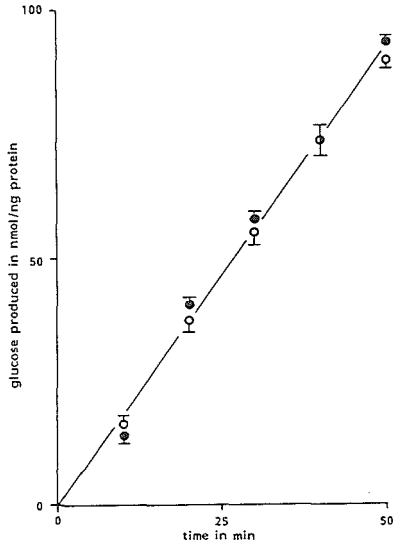

Figure 2. Influence of endotoxin on the glucose production by isolated parenchymal liver cells.

The cells were incubated in the absence (●) or presence (○) of 100 µg/ml LPS (026-B6). Data are mean ± S.D. of 3 experiments.

Because endotoxin has been reported to stimulate eicosanoid synthesis in various cell types like vascular endothelial cells, neutrophils, preoptic nerve cells and monocytes (17, 18), we investigated whether stimulation of liver prostaglandin synthesis by endotoxin was related to the stimulation of glucose-output. We therefore preperfused the liver with aspirin, a well-known irreversible cyclooxygenase inhibitor (19), in order to block the prostaglandin synthesis in the liver. The presence of aspirin completely blocked the effect of endotoxin on the glucose-output by the liver (Fig. 1). This suggests that an eicosanoid is in-

volved in the transduction of endotoxin induced stimulation of glycogenolysis in perfused liver. The eicosanoids probably originate from non-parenchymal liver cells, most likely Kupffer cells because Kupffer cells are known to produce several prostaglandins (19-21). Recently we established that the most prominent eicosanoid produced by Kupffer cells was prostaglandin D_2(9) and that prostanoids produced by isolated Kupffer and endothelial liver cells could enhance glucose output of isolated parenchymal liver cells by activation of glycogen phosphorylase (8). In Fig. 3 the effect of prostaglandin D_2 and LPS on the amount of glucose pro-

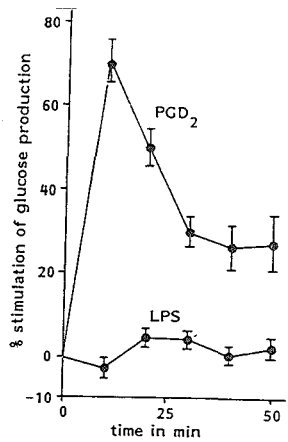

Figure 3. Influence of prostaglandin D_2 and endotoxin (LPS) on the glucose production by isolated parenchymal liver cells.

Prostaglandin D_2 (10^{-6} M) or endotoxin (LPS) (100 µg/ml) was added to isolated parenchymal liver cells. Data are mean ± S.E.M. of 3 experiments.

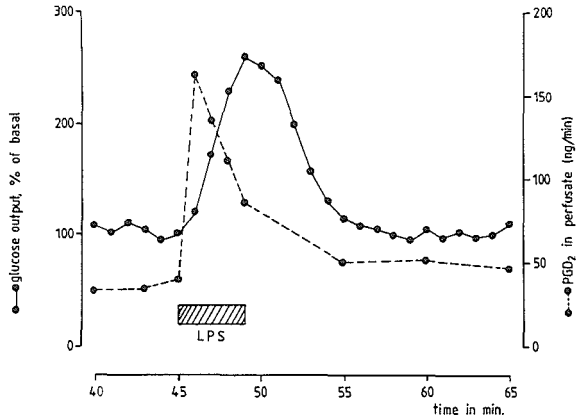

Figure 4. Influence of E.coli endotoxin (LPS) on prostaglandin D_2 and glucose production in the perfused liver.
Data are mean of a typical experiment out of 3.

duced by isolated parenchymal liver cells are compared. While prostaglandin D_2 stimulated the glucose production in isolated parenchymal cells up to 70%, LPS itself was ineffective.

To verify the hypothesis that prostaglandin D_2 mediates the effect of endotoxin on glycogenolysis we monitored in time the production of prostaglandin D_2 by the liver during endotoxin-stimulated glycogenolysis. Addition of endotoxin to the perfused liver induced a 5-fold increase in the rate of prostaglandin D_2 synthesis (Fig. 4), with a time course which preceeded the increase in glucose output. This indicates that the time course in prostaglandin D_2 release in the perfused liver allows a mediating role. The measured increase in prostaglandin D_2 production reflects most probably an underestimation of the total amount of prostaglandin D_2 formed in the liver, because prostaglandin D_2 in the perfused liver system is rapidly metabolized (23).

In conclusion we state that endotoxin stimulates glycogenolysis in the liver via an indirect mechanism, involving intercel-

lular communication. In this model, endotoxin specifically interacts with Kupffer cells (6), thereby triggering the production of prostaglandin D_2 (Fig. 4). Subsequently prostaglandin D_2 stimulates glycogen breakdown in parenchymal liver cells, which leads to increased glucose production by these cells. It was shown earlier (9) that prostaglandin D_2 is the major eicosanoid formed by Kupffer cells (55% of the total amount of formed prostanoids), while it proved to be the most effective prostaglandin in stimulating glucose production by parenchymal cells (8). However, it cannot be excluded that other prostanoids or a physiological combination of various prostanoids will contribute to the overall effect. The mechanism of intercellular communication may provide the carbohydrate source of energy necessary for the effective destruction of invaded microorganisms, by phagocytic cells including the Kupffer cells. Our model also explains the observed hyperglycemia in early or mild endotoxemia, while during prolonged endotoxemia the exhaustion of the liver's glycogen pool and/or and inadequate gluconeogenesis (22) may lead to hypoglycemia.

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