Lipoprotein Lipase Expression in Undifferentiated Hepatoma Cells Is Regulated by Progesterone and Protein Kinase A†

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ABSTRACT: Recently, it was shown that lipoprotein lipase (LPL) was produced in neonatal but not in adult rat liver. In an attempt to further define the mechanism involved in liver LPL expression, we identified a neonatal mouse hepatoma cell line, $BWTG_3$, capable of producing LPL. The regulation of LPL expression by various extracellular stimuli was investigated in this cell line. Progesterone caused a rise in LPL production by $BWTG_3$ cells. Other hormones tested, such as insulin, glucagon, adrenalin, testosterone, and thyroid hormone, had no effect on LPL production. The effects of progesterone on LPL production showed slow kinetics reaching a maximum 24 h after addition. Cotransfection of a progesterone receptor expression vector with a 5′-LPL–CAT reporter construct resulted in an induction of CAT activity, suggesting that the increase in LPL accumulation afer progesterone was linked to transcriptional induction of the LPL gene. Stimuli causing an elevation of protein kinase A activity in the cells also increased LPL production. Three agents capable of elevating intracellular cAMP levels, i.e., forskolin, dBcAMP, and choleratoxin, caused an elevation of LPL production. The increase in LPL activity caused by forskolin and choleratoxin was paralleled by an elevation of LPL mRNA levels, while dBcAMP only induced a small elevation of LPL mRNA levels. The increase in LPL production was shown to be linked to the stimulation of the PKA signal transduction pathway and was apparently transmitted via the transcription factor CREB. No effect of the stimulation of protein kinase C or calcium/calmodulin-dependent kinase on LPL production was detected. In conclusion, LPL production is regulated by progesterone and agents stimulating protein kinase A in $BWTG_3$ hepatoma cells, making these cells a valuable model to study the regulation of LPL production.

Lipoprotein lipase (LPL), a secreted enzyme, is responsible for the hydrolysis of triacylglycerols in plasma lipoproteins (Olivecrona & Bengston-Olivecrona, 1987; Garfinkel & Schotz, 1987; Brunzell, 1991). This reaction makes fatty acids available for use in cellular processes and occurs at the vascular bed of several extrahepatic tissues including heart and skeletal muscle, adipose tissue, breast, and lungs. LPL is produced by numerous tissues but not by adult liver (Garfinkel & Schotz, 1987). LPL is therefore considered an extrahepatic enzyme, but actually LPL activity can be detected in the liver under various conditions and in different species (Chajek et al., 1977; Vilaro et al., 1986). Although adult liver normally synthesizes hepatic lipase (HL) but not LPL, the synthesis of LPL in the liver of newborn rats was recently reported (Burgaya et al., 1989; Vilaro et al., 1988; Peinado-Onsurbe et al., 1992). Liver LPL synthesis is, however, extinguished after birth in a process influenced by thyroid and glucocorticoid hormones (Peinado-Onsurbe et al., 1992). In an attempt to further define the mechanism involved in liver expression of LPL, we used a hepatoma cell line, $BWTG_3$, which resembles undifferentiated neonatal hepatocytes (Szpirer & Szpirer, 1975) and was still capable of producing LPL. The goal of this paper was, therefore, to investigate the regulation of LPL production in vitro, using this cell line, and compare it with the regulation of liver LPL production in vivo. It is shown that LPL mRNA is present in $BWTG_3$ cells and that LPL activity is secreted in the medium. Steady-state LPL mRNA levels are significantly influenced by the addition of progesterone or by agents stimulating the protein kinase A signal transduction pathway. Addition of other agents stimulating the protein kinase C or calcium/calmodulin-dependent kinase had no effect on LPL production. Similarly, addition of other hormones, including various steroid hormones, thyroxine, insulin, or adrenalin, had no significant effects on LPL production.

MATERIALS AND METHODS

Materials. Progesterone (PG), thyroxine (T4), dexamethasone (DEX), testosterone (TES), forskolin (FORS), dibutyryl-cAMP (dBcAMP), A-23187, adrenalin (ADR), retinoic acid (RA), phorbol 12-myristate 13-acetate (PMA), coleratoxin (CT), dioctanyl-*rac*-glycerol (DAG), and glucagon (GLCN) were obtained from Sigma. Heparin and insulin were bought from Gibco/BRL, H-8 was from Seikaguchi, and 1,9-dideoxyforskolin (1,9-FORS) was from Calbiochem.

Cell Culture. The mouse hepatoma cell line $BWTG_3$ was a kind gift of Drs. Szpirer and Szpirer (Szpirer & Szpirer, 1975), while the HepG2 and THP-1 cells were obtained from the ATCC. Falcon tissue culture dishes were used. Cells were maintained in Dulbecco's modified Eagle's minimal essential medium (for the $BWTG_3$ and HepG2 cells) or RPMI-1640 (for THP-1), supplemented with 10% fetal calf serum at 37 °C in a humidified atmosphere of 5% CO_2/95% air

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(Auwerx et al., 1988, 1989). In a selected set of experiments, charcoal-treated fetal calf serum was used.

Transfections and Transient Expression Assays. $BWTG_3$ cells were transfected at 50–60% confluency in 10-cm dishes by the calcium phosphate coprecipitation procedure (10-h contact time). All transfections were performed with a mixture of plasmids which contained in addition to the reporter and expression vector 1 μg of the β-galactosidase expression vector complementing to a total amount of 15 μg of plasmid DNA. Cells were washed with PBS after 10 h, fresh medium containing 10% FCS with hormones was then added, and cells were harvested after 16 h. For some experiments, charcoal-stripped FCS was used. CAT activity was determined on cell extracts, after normalization to β-galactosidase activity, exactly as described by Gorman et al. (1982). Autoradiographs were quantified by densitometry. Transfection experiments were performed at least 4 times, and the results of a representative experiment are shown.

LPL Activity Measurements. Media were changed at the beginning of the experiments, and left in place until collection. In order to determine LPL activity, media were collected, chilled to 4 °C, and immediately centrifuged at low speed to remove possible cellular contamination. Media samples were then aliquoted, frozen in liquid nitrogen, and stored at −80 °C untill analysis. LPL activity was determined by the method of Ramirez et al. (1985). No interference of hepatic lipase activity was present since $BWTG_3$ cells did not contain hepatic lipase activity nor did they secrete any hepatic lipase activity (data not shown). The assay mixture contained 0.6 mM glycerol tri[9,10(N)-^{3}H]oleate (12 Ci/mol), 50 mM $MgCl_2$, 0.05% albumin (fatty-acid-free), 3% (v/v) serum (pretreated for 60 min at 50 °C), 25 mM Pipes (pH 7.5), and 0.02 mL of sample in a final volume of 0.2 mL. The incubation was carried out for 30 min at 25 °C. The reaction was terminated, and the released [^{3}H]oleate was quantified as previously described by Vilaro et al. (1986). One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of oleate per minute at 25 °C. All experiments involving LPL activity determinations were performed at least 6 times, and mean values ± SEM are reported. In all of the experiments involving LPL activity determinations (except the experiments reported in Figure 2), heparin was added to the media of both control and treated cells to enhance LPL stability. The difference in LPL activity between the control and the stimulated cells was therefore taken as the effect specifically induced by the stimulus.

Recombinant Plasmids. Classical recombinant technology was used (Maniatis et al., 1982). pHLPL-26 contains a human LPL cDNA clone (Auwerx et al., 1988; Deeb & Peng, 1989). Genomic restriction fragments containing various lengths of the LPL 5′-upstream sequences were subclones of clone B-1 described in Deeb and Peng (1989). The LPL 5′-upstream sequences were cloned into the polylinker of the promotorless expression vector KS$^+$-SV0-CAT (Tsonis et al., 1988). KS$^+$-PN-CAT contained a fragment spanning −1719 to +136, KS$^+$-XN-CAT a fragment from −852 to +136, and KS$^+$-AN-CAT a fragment from −230 to +136 of the LPL genomic sequence [(−) refers to 5′-upstream sequences as defined in Deeb and Peng (1989)]. All these constructs were examined by restriction mapping and partial sequencing. hPR1, hPR2, MMTV-CAT, and PRE/GRE-tk-CAT were a kind gift of Dr. P. Chambon (Strasbourg) and are described in Kastner et al. (1990). The CREB expression vector is a gift from Dr. Paolo Sassone-Corsi (Strasbourg), while the PKA expression vector was a kind gift from Dr. S. McKnight (Seattle, WA; Orellana & McKnight, 1990). KS$^+$-SV0-CAT was used as a negative control, while KS$^+$-SV2-CAT, containing the SV-40 promoter and a CMV-driven β-galactosidase plasmid, served as a positive control and a control for transfection efficiency, respectively, in transfections. MMTV-CAT and PRE/GRE-tk-CAT constructs were used to analyze the effect of hPR1 and hPR2 cotransfections.

Analysis of RNA. RNA from cells was isolated by the guanidine isothiocyanate method and analyzed either by electrophoresis through 1.2% agarose–formaldehyde gels followed by capillary transfer to nylon membranes or by dot blot hybridization as described (Auwerx et al., 1988). Transcripts were quantitated by densitometric tracing of autoradiographs following hybridization to ^{32}P-labeled probes. A 1.36-kb *Eco*RI fragment of the human LPL cDNA clone (hLPL-26) (Auwerx et al., 1988) was labeled by the random priming technique. A human apo E and a chicken β-actin clone were used as controls (Auwerx et al., 1988). LPL values were corrected for variations in β-actin. Experiments involving RNA quantifications were performed at least twice in an independent fashion, and a representative experiment was shown.

For analysis of RNA by the polymerase chain reaction (PCR), 1 μg of total RNA was reverse-transcribed for 45 min at 37 °C by using 100 units of Moloney murine leukemia virus reverse transcriptase and 5 pmol of HLPL-9C (5′CTTCACAGAATTCACATGCC-3′) as primer. One-tenth of the resulting cDNA mixture was amplified in the presence of 200 μM dNTP's, 10 pmol of both HLPL-9C and HLPL-8 (5′-ATGGAAGAGTGATTCATACT-3′) primer, and 1 unit of Taq polymerase as specified by the supplier (Perkin-Elmer-Cetus). The reaction mixture was denatured at 94 °C for 1 min, annealed at 56 °C for 1 min, and extended at 72 °C for 1 min for a total of 30 cycles. A fraction of the PCR mixture was analyzed on an ethidium bromide-stained 1.5% agarose gel.

Statistical Methods. Analysis of variance (ANOVA) was used to evaluate the results of the dose response and time course experiments. Values observed between different gruops were compared by contrast statements. A two-tailed unpaired Student's *t* test was used to evaluate differences between means in all other experiments.

RESULTS

LPL mRNA and LPL Activities Are Present in $BWTG_3$ Liver Cells. Since in vivo substantial amounts of LPL can be detected in the neonatal liver, we decided to investigate LPL production by liver cell lines in more detail. Therefore, we tested both human hepatoblastoma cell lines, HepG2 and Hep3B, which have some differentiated characteristics, and the mouse hepatoma line $BWTG_3$, which is much less differentiated, for the presence of LPL mRNA and activity. In HepG2 and Hep3B, no LPL mRNA could be detected either by Northern blot RNA hybridization (Figure 1B) or by the sensitive technique of PCR amplification of cDNA synthesised from HepG2 mRNA (Figure 1A). Consequently, no LPL activity could be detected in the medium of HepG2 or Hep3B cells. In contrast, $BWTG_3$ cells showed a considerable amount of LPL mRNA (Figure 1B). The presence of LPL synthesis in $BWTG_3$ cells could furthermore also be deduced from the detection of LPL activity in the culture medium of $BWTG_3$ cells (Figure 2). Interestingly, contrary to HepG2 and Hep3B cells, $BWTG_3$ cells did not contain any hepatic lipase mRNA nor did they secrete hepatic lipase activity into the medium (data not shown).

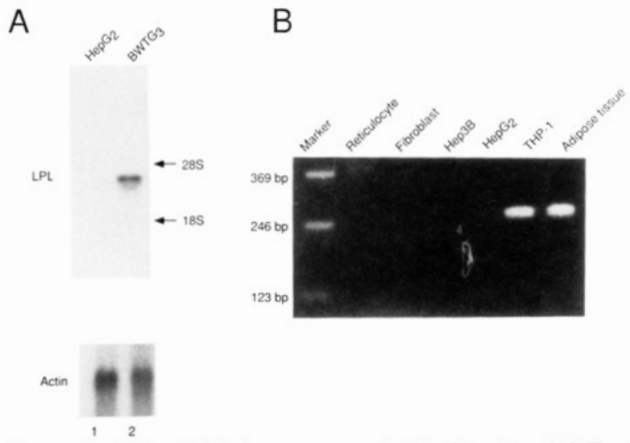

FIGURE 1: LPL mRNA is not present in HepG2 cells. (A) Total RNA isolated from several human cells were reverse-transcribed. The resulting cDNA was amplified by PCR as described and analyzed on a 1.5% agarose gel. Size markers are indicated in the margin. The amplification product specific for LPL mRNA is visible in lanes containing material originating from the PMA-induced human monocytic leukemia cell line THP-1 and from human adipose tissue. (B) Northern blot of 20 μg of total $BWTG_3$ RNA hybridized with the LPL cDNA. Hybridization of the same blot with β-actin cDNA is shown as a control. The size of the 28S and 18S ribosomal RNAs is indicated.

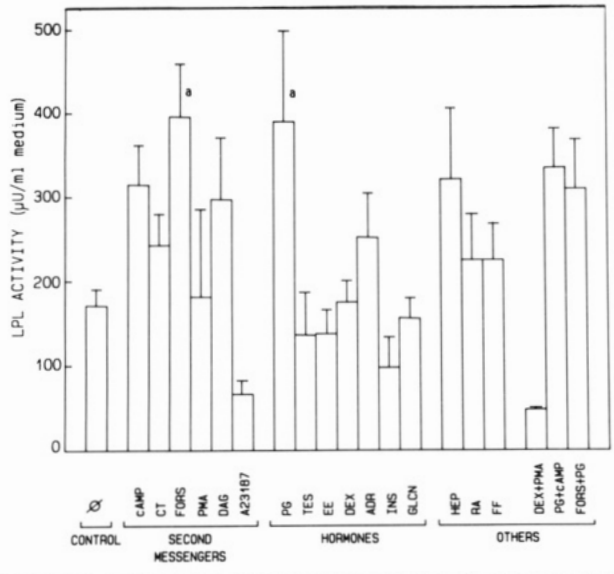

FIGURE 2: LPL mRNA is present in $BWTG_3$ cells and regulation of LPL activity in these cells. Confluent $BWTG_3$ cells were incubated for 24 h with the following stimuli: dBcAMP (100 μM); cholera toxin (CT, 10 ng/mL); forskolin (FORS, 25 μM); PMA (1.6×10^{-6} M); DAG (20 μg/mL); A23187 (5 μg/mL); progesterone (PG, 1 μg/mL); testosterone (TES, 5×10^{-3} M); ethinylestradiol (EE, 1 μM); dexamethasone (DEX, 10 μM); adrenalin (ADR, 2 μM); insulin (INS, 100 μg/mL); glucagon (GLCN, 0.1 μM); heparin (HEP, 10 units/mL); retinoic acid (RA, 10^{-6} M); fenofibrate (FF, 1 mM); dexamethasone + PMA: progesterone + dBcAMP; and progesterone + forskolin. It needs to be stressed that the concentrations of the various stimuli used represent the highest concentrations we tested. In some experiments, lower concentrations of stimulating agents were used, but the effects were comparable to the effects obtained with higher amounts of stimulating agents. Media were collected after each experiment and analyzed for LPL activity as described. The result represents the mean ± 1 SEM of seven independent experiments. Statistical differences are observed between values followed by different letters.

Regulation of LPL Synthesis in $BWTG_3$ Cells by Stimuli of Signal Transduction Pathways and Hormones. Since in vivo liver LPL expression is strongly regulated, we next investigated if LPL activity and mRNA levels in $BWTG_3$ cells were also influenced by extracellular stimuli, such as provided by hormones or stimuli activating the various second-messenger pathways (Figure 2). All agents which stimulate the cAMP-dependent protein kinase A (PKA) pathway induced LPL activity in $BWTG_3$ cells, although only the effect of forskolin was significant in this experiment. Addition of forskolin (FORS, 25 μM) doubled LPL activity in the medium, and dibutyryl-cAMP (dBcAMP, 100 μM) caused a 70% increase, while cholera toxin (CT, 10 ng/mL) caused a more modest increase in LPL activity. Stimulation of the protein kinase C (PKC) signal transduction pathway by the addition of either the phorbol ester phorbol 12-myristate 13-acetate (PMA, 1.6×10^{-7} M) or the diacylglycerol analog dioctanyl-*rac*-glycerol (DAG, 20 μg/mL) to the cells had no consistent effect on LPL activity in the medium. These agents furthermore did not cause any alteration in LPL mRNA steady-state levels (data not shown). An increase in intracellular calcium induced by the addition of the calcium ionophore A-23187 (5 μg/mL) caused a decrease in LPL activity (Figure 2) without an effect on LPL mRNA levels (not shown). This decrease in LPL activity, could, however, be ascribed to a general cell toxicity induced by this agent.

Several hormones, including progesterone (PG, 1 μg/mL), testosterone (TES, 5×10^{-3} M), ethinylestradiol (EE, 1 μM), dexamethasone (DEX, 10 μM), adrenaline (ADR, 2 μM), insulin (INS, 100 μg/mL), and glucagon (GLCN, 0.1 μM), were also tested for a potential effect on LPL activity. To our surprise, only progesterone caused a significant alteration in LPL activity or LPL mRNA, inducing a 2-fold rise in the LPL activity in the medium and a 3-fold increase in LPL mRNA. Combined addition of progesterone with stimulators of the PKA signal transduction pathway, such as dBcAMP or forskolin, did not cause any further increase in LPL mRNA (data not shown) or in LPL activity detected in the medium of $BWTG_3$ cells relative to when each agent was added individually (Figure 2).

Heparin (HEP, 10 units/mL), which is known to stabilize LPL activity, also induced an increase in LPL activity measured in the medium without affecting the LPL mRNA levels (data not shown). The morphogen retinoic acid (RA, 10^{-6} M) and the drug fenofibrate (FF, 1 mM) had no significant effect on either LPL activity (Figure 2) or mRNA levels (not shown) in this cell line.

LPL Gene Transcription Is Induced by Progesterone. Consequently, the effects of the addition of progesterone to the tissue culture medium were analyzed in more detail. Progesterone activates a receptor of the steroid hormone receptor superfamily (Green & Chambon, 1986; Evans, 1988). The progesterone receptor is a phosphoprotein that transduces the signal carried by progesterone resulting in the regulation of gene expression. Progesterone addition for 24 h to $BWTG_3$ cells caused a dose-dependent increase in LPL activity in the medium. At a concentration of 10^{-2} μg/mL, LPL activity doubled while at concentrations of 1 or 10 μg/mL the amount of LPL activity accumulating in the media almost increased by 300% (Figure 3A). Addition of progesterone at a concentration of 1 μg/mL for variable periods of time showed that LPL activity initially briefly declined relative to a control culture (Figure 3B). Enhanced accumulation of LPL activity started between 6 and 12 h, reaching a maximum approximately 24 h after addition of progesterone (Figure 3B). This pattern of accumulation was confirmed in several independent experiments. Also, LPL mRNA levels showed a time-dependent increase (Figure 3B,C). The increase in LPL mRNA levels could first be observed 12 h after addition of progesterone. Accumulation of LPL continued until 72 h,

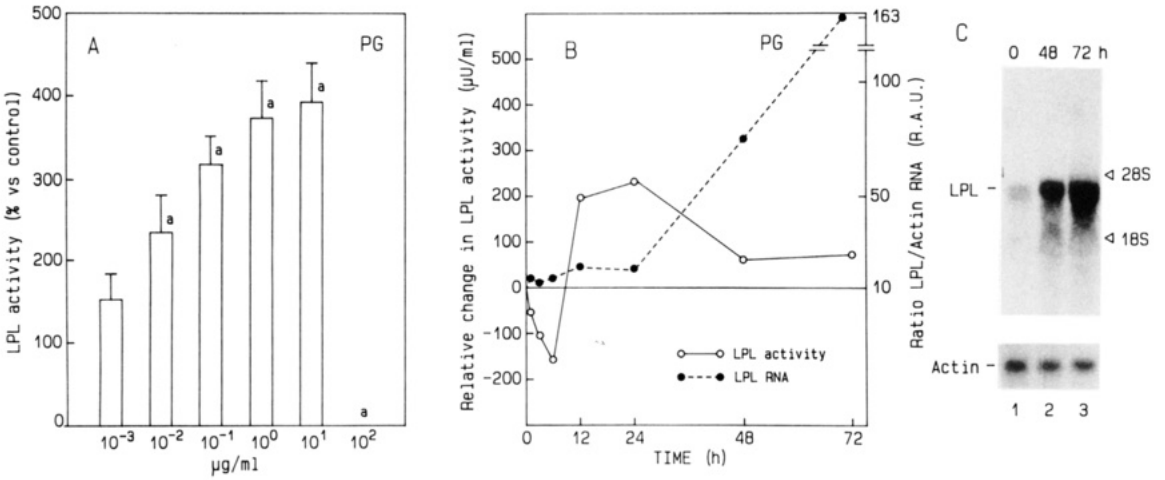

FIGURE 3: Progesterone causes a time- and dose-dependent increase in $BWTG_3$ LPL production. (A) Dose response of LPL activity for progesterone. Confluent $BWTG_3$ cells were incubated in triplicate for 24 h with progesterone at the indicated concentrations. LPL activity was determined as described. Results represent the mean ± 1 SEM. (B) Time course of LPL activity and mRNA accumulation. Confluent $BWTG_3$ cells were incubated for the incubated time with progesterone (1 μg/mL) or solvent alone. LPL activity determinations were performed on triplicate dishes. The LPL activity value represents the difference between the mean LPL activity of the stimulated cells from that of the vehicle-treated cells. LPL activity in control cultures showed a slow increase with time. LPL mRNA values were determined by dot blot hybridization as described under Materials and Methods. The LPL mRNA curve represents the ratio obtained by correcting the relative absorbance for LPL mRNA relative to the β-actin mRNA. (C) Northern blot showing the effect of progesterone on $BWTG_3$ LPL mRNA levels. RNA obtained before or 48 h after addition of progesterone to the culture medium was analyzed by Northern blot hybridizations as described. The size of the 28S and 18S RNA is indicated. Hybridization of the same blot with β-actin cDNA is shown as a control.

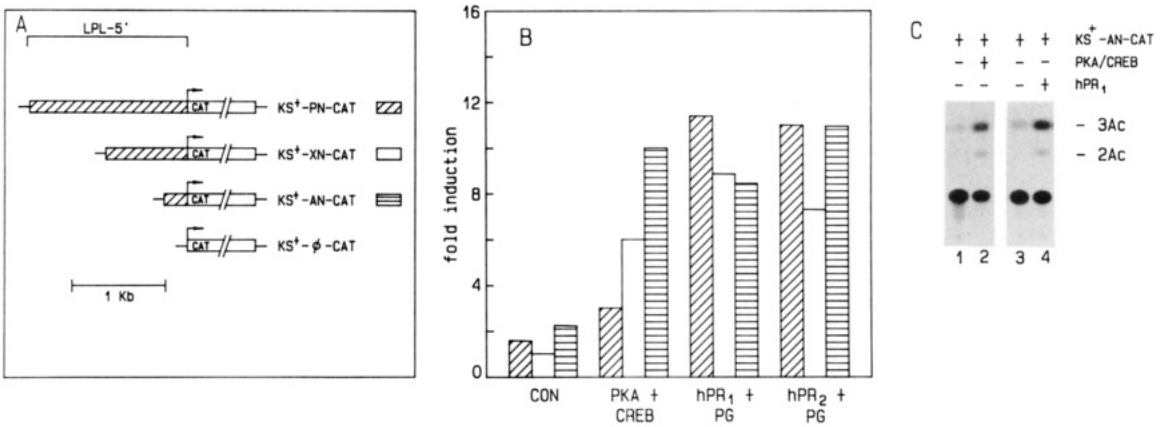

FIGURE 4: Induction of LPL mRNA is transcriptional. (A) Structure of LPL–CAT constructs. Various fragments of the LPL 5′-upstream regulatory region, representing regions from −1719 to +136, from −852 to +136, and from −230 to +136, were cloned in the vector KS+-0-CAT and used as reporter constructs. The boxes to the right to the names of the constructs correspond to the symbols used in (B). (B) Effect of cotransfection of expression vectors on LPL–CAT reporter constructs. LPL–CAT reporter constructs were cotransfected with either the PKA/CREB, the hPR1, or the hPR2 expression vectors. The amount of CAT activity obtained from these constructs relative to the KS+-0-CAT construct is plotted. A somatostatin–CRE–CAT construct was induced 53-fold by cotransfection with PKA and CREB expression vectors is similar conditions. The MMTV-CAT construct was 56- 60-fold while the PRE/GRE-tk-CAT construct was 73- and 89-fold induced by hPR1 and hPR2, respectively. The results are representative of at least four independent experiments. (C) Representative CAT assay. The induction of KS+-AN-CAT (lane 2) by PKA/CREB and hPR1 (lane 4) is depicted relative to basal KS+-AN-CAT expression (lanes 1 and 3).

when LPL mRNA levels were 16-fold higher than before the addition of progesterone while LPL activity was already beyond its maximal level. A Northen blot hybridization experiment illustrating this effect is shown in Figure 3C. Since the previously mentioned experiments were performed in the presence of 10% fetal calf serum, which contains endogenous steroids, we also tested whether progesterone (10^{-2} μg/mL) added to cells incubated with charcoal-treated fetal calf serum was also effective in increasing LPL mRNA and activity levels. In fact, essentially the same amount of induction (3-fold increase in LPL activity and a 12-fold increase in LPL mRNA steady-state levels) was obtained under these conditions (data not shown), demonstrating the validity of our previous experiments.

Next it was investigated if the increase in LPL mRNA levels in response to progesterone was caused by an induction of transcription of the LPL gene. In view of the low transcription rates for the LPL gene in hepatoma cells, we have chosen to utilize a (co)transfection strategy. Therefore, we transfected plasmid reporter constructs, in which nested deletions of the 5′-LPL regulatory sequences were driving the expression of the chloramphenicol acetyltransferase (CAT) reporter gene, with or without expression vectors coding for the human progesterone receptor forms A and B (hPR1 and hPR2) (Kastner et al., 1990). When cells were maintained in regular medium containing 10% FCS, all successive nested deletions (up to KS+-AN-CAT; Figure 4A) were induced upon hPR1 or hPR2 cotransfection, resulting in a 4–6-fold increase in accumulation of CAT activity in the cells (Figure 4B). This effect was suggestive for a transcriptional effect mediated through the LPL promoter. No effect of cotransfection of hPR1 or hPR2 could be detected on the promotorless

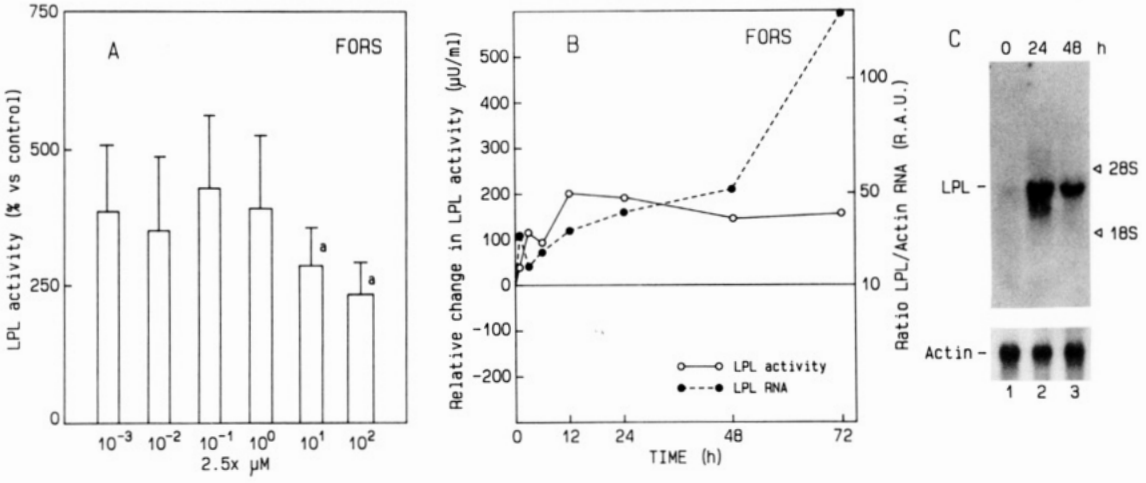

FIGURE 5: Forskolin causes a time- and dose-dependent increase in $BWTG_3$ LPL production. (A) Dose response of LPL activity for forskolin. Confluent $BWTG_3$ cells were incubated in triplicate for 24 h with forskolin at the indicated concentrations. LPL activity was determined as described. Results represent the mean ± 1 SEM. (B) Time course of LPL activity and mRNA accumulation. Confluent $BWTG_3$ cells were incubated for the incubated time with forskolin (25 μM) or solvent alone. LPL activity determinations were performed on triplicate dishes. The LPL activity value represents the difference between the mean LPL activity of the stimulated cells from that of the vehicle-treated cells. LPL activity in control cultures showed a slow increase with time. LPL mRNA values were determined by dot blot hybridization as described under Materials and Methods. The LPL mRNA curve represents the ratio obtained by correcting the relative absorbance for LPL mRNA relative to the β-actin mRNA. (C) Northern blot showing the effect of forskolin on $BWTG_3$ LPL mRNA levels. RNA obtained before or 48 h after addition of forskolin to the culture medium was analyzed by Northern blot hybridizations as described. The size of the 28S and 18S RNA is indicated. Hybridization of the same blot with β-actin cDNA is shown as a control.

KS^+-0-CAT or the KS^+-SV2-CAT construct (not shown), while the MMTV-CAT was induced 56- and 60-fold and the PRE/GRE-tk-CAT construct was induced 73- and 89-fold by hPR1 and hPR2, respectively. When the LPL-CAT constructs were transfected, without cotransfection of the progesterone receptor, into cells maintained in medium supplemented with charcoal-treated serum, a 3-fold induction of CAT activity could be detected after addition of 10^{-2} μg/mL progesterone (data not shown). Under these conditions, a strong induction of the MMTV-CAT and PRE/GRE-tk-CAT was still evident, suggesting that these cells contain a progesterone receptor.

LPL Is Also Induced by Agents Stimulating Protein Kinase A. Since not only progesterone but also several agents which increase intracellular cAMP levels increased LPL activity secreted in the $BWTG_3$ medium (Figure 2), we investigated the effects of several stimuli of PKA activity in more detail. Forskolin, an activator of adenylate cyclase, was capable of provoking an increase in LPL activity in the medium (Figure 5A). The increase in LPL activity was already at its maximal level at a concentration of forskolin of 0.25 μM. Due to experimental variation, however, only the values obtained after 2.5–2.50 μM forskolin caused a statistically significant increase in LPL activity. The effect of 25 μM forskolin on LPL activity accumulation in the medium increased upon prolongation of the incubation period (Figure 5B). LPL activity started accumulating very shortly after forskolin addition (1 h) and reached a maximal level 12–24 h after addition. This increase in LPL activity observed in the medium was associated with a strong increase in the steady-state levels for LPL mRNA, which were already elevated 1 h after addition but continued to rise until 72 h, when a 12-fold induction of LPL mRNA was evident (Figure 5B,C).

Forskolin has been studied most extensively for its effects on adenylate cyclase, but it has many other effects on the cells as well (Laurenza et al., 1989). Since forskolin has pleiotropic effects on cellular metabolism, we wanted to establish whether this increase in LPL activity was associated with activation of the cAMP/PKA signal transduction pathway. Other agents capable of raising the intracellular cAMP concentration were therefore tested for a potential effect on LPL accumulation in the medium. Cholera toxin (CT), an agent which irreversibly ADP-ribosylates the α-subunit of the G_S protein, causes a strong activation of adenylate cyclase with consequent cAMP accumulation in the cell as a result. Interestingly, CT also induces LPL activity in $BWTG_3$ cells (Figure 6A,C). The induction by cholera toxin requires a minimum concentration of 0.1 ng/mL (Figure 6A). LPL activity in the medium showed an initial decrease early (6 and 12 h) after addition of 10 ng/mL CT. At later time points (24 and 48 h), LPL activity accumulated, however, to a greater extent than in control cultures. LPL mRNA levels increased 4-fold. The increase in LPL mRNA corresponded with the LPL activity accumulation (Figure 6C).

dBcAMP, a cAMP analog which easily penetrates the cell membrane, was also capable of inducing LPL, activity, when added at a final concentration of 10 mM (Figure 6B,D). The effect of dBcAMP on LPL activity accumulation (Figure 6B) displayed kinetics different from the time course observed after the addition of forskolin (Figure 5). LPL activity dropped slightly after an initial rise (after 1 and 3 h) and accumulated again to levels much higher after 24 h. dBcAMP only increased LPL mRNA levels 2-fold relative to actin mRNA (Figure 6D).

Since three different stimuli activating the PKA signal transduction pathway activate LPL activity, we also investigated the effect of inhibitors of PKA on LPL production. The effect of the PKA inhibitor *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride or H-8 was first investigated (Figure 7A). When added by itself, H-8 was capable of suppressing LPL accumulation in the medium. Furthermore, H-8 could inhibit the forskolin-induced rise in LPL activity in $BWTG_3$ medium. A naturally occurring forskolin analog, 1,9-dideoxyforskolin (1,9-FORS), which does not activate adenylate cyclase, but which reproduces many of the cAMP-independent effects of forskolin [reviewed by Laurenza et al. (1989)], was tested next. As expected, 1,9-dideoxyforskolin showed no effect on LPL accumulation in the medium, suggesting that the forskolin effect was indeed linked to activation of adenylate cyclase (Figure 7B).

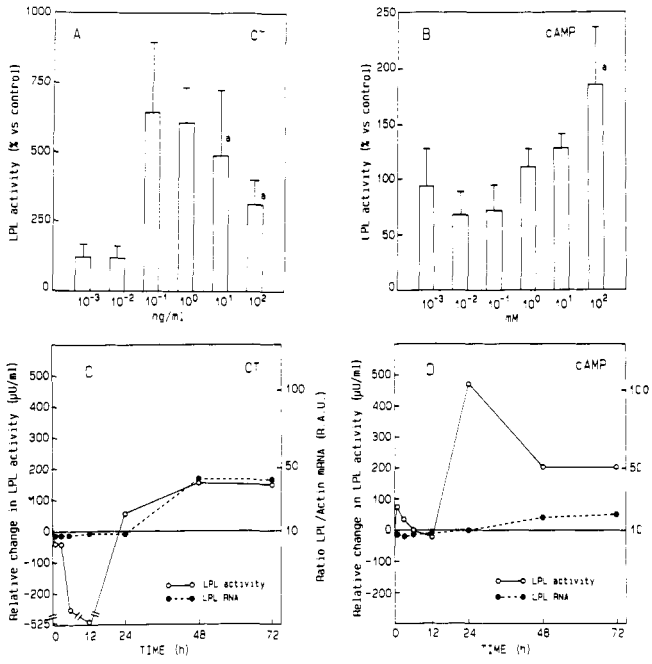

FIGURE 6: CT or dBcAMP also causes time- and dose-dependent increase in $BWTG_3$ LPL production. (A and B) Dose response of LPL activity for CT (A) and dBcAMP (B). Confluent $BWTG_3$ cells were incubated in triplicate for 24 h with either CT or dBcAMP at the indicated concentrations. LPL activity was determined as described. Results represent the mean ± 1 SEM. (C and D) Time course of LPL activity and mRNA accumulation. Confluent $BWTG_3$ cells were incubated for the incubated time with CT (C, 10 ng/mL) or dBcAMP (D, 100 μM) or solvent alone. LPL activity determinations were performed on triplicate dishes. The LPL activity value represents the difference between the mean LPL activity from the stimulated cells with the vehicle-treated cells. LPL activity in control cultures showed a slow increase with time. LPL mRNA values were determined by dot blot hybridization as described under Materials and Methods. The LPL mRNA curve represents the ratio obtained by correcting the relative absorbance for LPL mRNA relative to the β-actin mRNA.

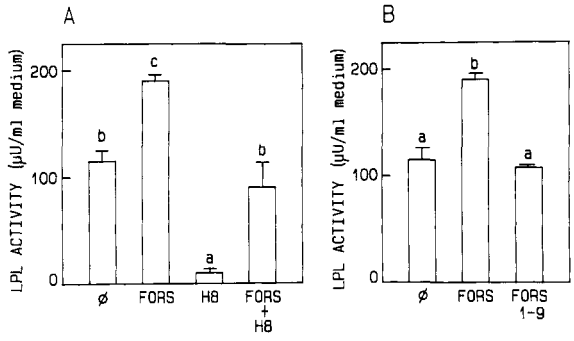

FIGURE 7: PKA is directly implicated in LPL induction in $BWTG_3$ cells. (A) LPL production is inhibited by inhibition of PKA. $BWTG_3$ cells were incubated during 24 h with vehicle alone (ϕ), forskolin (FORS), H-8 alone (H8), or H-8 and forskolin in combination (FORS + H8). Media were collected and analyzed for LPL activity. The results represent the mean ± 1 SEM from three independent experiments. (B) A forskolin analog lacking PKA stimulatory effects cannot induce LPL activity. $BWTG_3$ cells were incubated during 24 h with vehicle alone (ϕ) forskolin (FORS), or 1,9-dideoxyforskolin (FORS 1-9). Media were collected and analyzed for LPL activity. The results represent the mean ■ 1 SEM from three independent experiments.

We next wanted to investigate whether the effects of stimulation of the PKA signal transduction pathway on LPL were at the transcriptional level. It has been shown that cAMP regulates transcription of eukaryotic genes through the reversible PKA-dependent phosphorylation of a target nuclear protein, the transcription factor CREB (Yamamoto et al., 1988; Hoeffler et al., 1988). Therefore, the 5′-LPL–CAT reporter constructs (Figure 4A) were cotransfected with two expression vectors, one coding for the catalytic subunit of PKA and another coding for the transcription factor CREB. As expected, the 5′-LPL–CAT constructs were induced 3–6-fold by cotransfection with both PKA and CREB (Figure 4B), with the constructs containing smaller parts of the 5′-URS consistently induced to a higher extent. When only the PKA expression vector was cotransfected, a very weak induction of CAT activity was observed, while when the CREB expression vector was cotransfected with the LPL–CAT constructs a 2-fold induction was detected (not shown). This suggested that stimulation of the PKA signal transduction pathway can induce the LPL gene via the transcription factor CREB and suggests that this transcriptional induction might explain the increase in LPL activity and mRNA.

DISCUSSION

Although the liver usually does not produce LPL, we recently identified substantial amounts of LPL activity (Burgaya et al., 1989; Llobera et al., 1979; Vilaro et al., 1988; Peinado-Onsurbe et al., 1991) and LPL mRNA (Peinado-Onsurbe et al., 1992) in the liver of neonatal rats. This LPL activity and LPL mRNA disappeared gradually during development in a process remarkably similar to the neonatal extinction of α-fetoprotein (AFP) in the liver (Peinado-Onsurbe et al., 1992). The extinction processes, occurring in vivo, could be influenced by variations in food intake, hormonal changes (i.e., alterations in thyroid and glucocorticoid hormone levels), and administration of drugs such as fibrates (Peinado-Onsurbe et al., 1992; Staels & Auwerx, 1992). To analyze the regulation of liver LPL production in vitro, we identified a cell line capable of expressing large amounts of LPL, i.e., the mouse hepatoma cell line $BWTG_3$ (Szpirer & Szpirer, 1975). This $BWTG_3$ cell line represents a useful model to study undifferentiated hepatic cells. In contrast to relatively differentiated cell lines, such as the human hepatoblastoma line HepG2 and the rat hepatoma line Fa32, which already secrete albumin, $BWTG_3$ cells do not yet express albumin and still produce several immature markers, most notably AFP. In this study, we investigated events which influence the LPL gene in this undifferentiated hepatoma cell line. Two major external stimuli (which were apparently different from the factors influencing liver LPL production in vivo) seemed to be capable of altering hepatocyte LPL production in vitro, i.e., stimulation of the progesterone receptor and stimulation of the PKA signal transduction pathway.

Progesterone induces LPL mRNA and LPL activity levels most likely via transcriptional induction of the LPL gene. Interestingly, the concentration of progesterone necessary to observe this effect was higher than the concentrations commonly required for most progesterone receptor-mediated responses. The evidence available at present therefore does not allow us to distinguish between a direct or indirect effect of progesterone and/or the progesterone receptor on LPL transcription. Indeed, the activated progesterone receptor could induce or activate an intermediary transcription factor, which will then in turn enhance LPL gene expression. Further study will, however, be needed to analyze this in more detail. Limited information is available upon the regulation of LPL production by progesterone in other cells. In the preadipocytic 3T3-L1 cell line, progesterone has been shown to be capable of augmenting intracellular LPL (Spooner et al., 1979). It was, however, not investigated at which level progesterone caused this induction in LPL activity.

Not only progesterone but also stimulation of the cAMP-dependent PKA signal transduction system caused an induction of LPL activity. All agents tested, including forskolin, CT, and dBcAMP, induced LPL activity. This effect appears to be specific since inhibition of PKA by the inhibitor H-8 abolishes the forskolin-induced increase in LPL activity and since a forskolin analog, which is incapable of activating the cAMP/PKA signal transduction pathway, did not have an effect on LPL production. The induction of LPL activity by the cAMP/PKA signal transduction system is most likely caused by a transcriptional induction of the LPL gene since it was demonstrated by the cotransfection experiments that PKA-dependent phosphorylation of the transcription factor CREB (Yamamoto et al., 1988; Hoeffler et al., 1988) is capable of inducing the 5′-LPL–CAT constructs. The increase in LPL activity was in fact parralleled by a rise in LPL mRNA levels after forskolin and CT. The induction in LPL activity by dBcAMP was, however, not matched by the limited induction of LPL mRNA steady-state levels. We have no explanation for the limited increase in LPL mRNA relative to the induction of LPL activity. It is, however, likely that induction of LPL activity by stimulation of the PKA system is only in part explained by a transcriptional effect. The PKA signal transduction system might also be involved in posttranscriptional and posttranslational processes such as protein modification and secretion. Another issue of importance is whether the effects of PKA and progesterone are independent. Indeed, it has been recently demonstrated that activation of the PKA signal transduction pathway can enhance progesterone receptor-enhanced transcription (Denner et al., 1990). It is unclear, however, whether progesterone and cAMP are targetting LPL gene transcription by a common effector. In this scenario, stimulation of PKA might have an effect on LPL production via phosphorylation of the progesterone receptor. We believe this hypothesis is, however, less likely in view of the absence of a strong response of the 5′-LPL–CAT constructs to cotransfection of the PKA expression vector.

Also in other cells and tissues, agents inducing PKA activity have been reported to modulate LPL production and secretion. In muscle and heart, the situation seems to be complex. Intracellular increases in cAMP concentration in cardiomyocytes or myocytes caused either an increase (Palmer et al., 1990), no effect (Cryer et al., 1981), or actually a decrease (Friedman et al., 1983) in LPL production. Expression of LPL mRNA in the rat heart is however, clearly induced after the administration of CT to rats, but appears to be localized in interstitial cells rather than in cardiomyocytes (Stein et al., 1991). In adipocytes, the situation is more straightforward since agents which increase cAMP levels have been shown to unequivocally decrease LPL in adipocytes and adipose tissue [reviewed by Eckel (1987)]. In contrast, our preliminary experiments in macrophage cell lines, such as THP-1, suggested that elevations in intracellular cAMP levels increased LPL production (unpublished results). PKA-dependent regulation of LPL production in hepatocytes and THP-1 cells appears therefore to be functioning in a fashion directly opposite to the adipocytes, suggesting strong tissue-specific differences in the regulation in LPL production. Further study will be necessary to find out if this effect is caused by tissue-specific transcriptional regulation of the LPL gene or another effect.

One constant observation detected after addition of both progesterone and agents inducing PKA was the relative discrepancy between LPL mRNA accumulation (which continued to increase at later time points) and LPL activity accumulation (which peaked around 24 h after addition of the stimulus). One needs to keep in mind that LPL measurements in the media reflect a combination of the effects of the various stimulating agents on synthesis, secretion, and degradation of LPL (Vannier et al., 1989; Vannier & Ailhaud, 1989). Posttranscriptional and posttranslational phenomena together with the extreme instability of the secreted LPL protein (and hence also activity) in the cell culture medium can hence explain this discrepancy between LPL activity and LPL mRNA. Similar discrepancies between LPL synthesis and secretion have been observed previously (Ong et al., 1989; Semenkovich et al., 1989; Semb & Olivecrona, 1989; Cisar et al., 1989; Vannier & Ailhaud, 1989).

More interesting are, however, the in vivo implications of the regulation of liver LPL production by progesterone and PKA. The progesterone receptor belongs to the superfamily of nuclear receptors (including those for steroid and thyroid hormones, and retinoids) whose members coordinate development and homeostasis in response to the binding of their ligands (Green & Chambon, 1986; Evans, 1988). The regulatory response to receptors of this class can be influenced by the concentration of nuclear receptor or the concentration of its ligand. During pregnancy, serum progesterone levels increase dramatically, initially produced by the corpus luteum and later by the placenta. It is therefore tempting to speculate that the elevated progesterone levels might activate the progesterone receptor, which in turn might cause the elevated fetal and neonatal liver LPL production (Burgaya et al., 1989; Vilaro et al., 1988). Also, the cAMP system appears to be strongly implicated in developmental regulation of gene expression in eukaryotes. In fact, recently the group of Schutz and Fournier (Boshart et al., 1990; Ruppert et al., 1990) reported a nice example of the role of cAMP in developmental regulation of gene expression in the liver. It was shown that interference with the cAMP signal transduction pathway was responsible for extinction of the liver-specific gene tyrosine aminotransferase (TAT) in microcell hybrids between hepatocytes and fibroblasts. The tissue-specific extinguisher (*Tse-1*) responsible for this effect was identified as the regulatory subunit RI-α of PKA (Boshart et al., 1991; Jones et al., 1991). It is therefore not excluded that the regulation of LPL gene expression by the PKA signal transduction pathway might be involved in the fascinating developmental extinction of the liver production of LPL. Further studies pertaining to the developmental implications of progesterone and PKA are currently underway.

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