CHANGES IN mRNA LEVELS OF INTRACELLULAR FATTY ACID METABOLISM REGULATORS IN HUMAN HEPATOMA HepG2 CELLS FOLLOWING THEIR TREATMENT WITH NON-ESTERIFIED FATTY ACIDS AND DEHYDROEPIANDROSTERONE

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Received: October 3, 2005; Accepted (with revisions): November 14, 2005

Key words: Fatty acids/Protein kinase C (PKC)/Liver fatty acid binding protein (L-FABP)/Insulin resistance/HepG2 cells

The effects of non-esterified fatty acids (NEFA) and hormone dehydroepiandrosterone (DHEA) on the levels of mRNAs of protein kinase C (PKC) - δ and - ϵ isoforms and those of liver fatty acid binding protein (L-FABP) were investigated in the human hepatoma HepG2 cell line. The cells were kept in low-serum, low-albumin medium during experiments. Low FA levels (100 μ M) and time intervals of 4 h and 20 h were used. In these conditions, the saturated (palmitic, stearic) and monounsaturated (oleic) acids rather selectively stimulated PKC- ϵ mRNA levels. Unexpectedly, we found that these acids also suppressed liver fatty-acid binding protein (L-FABP) mRNA levels. DHEA in pharmacological doses (100 μ M) produced a significant increase in PKC- δ and - ϵ mRNA levels. Although molecular mechanisms underlying the identified changes have not been investigated in this paper, our findings emphasize that NEFA-induced modulation of mRNA levels of key signalling components represent an additional mechanism for how the ambient NEFA can influence metabolic homeostasis in cells.

INTRODUCTION

The mechanisms by which ambient non-esterified fatty acids (NEFA) alter gene expression in cells are largely unknown. In fact, there are very different routes for FA to regulate a variety of genes (reviewed in^{1,2}). Significantly, the protein kinase C (PKC) family of intracellular serine/threonine protein kinases that plays a prominent role in a number of signalling pathways regulating cell metabolism, growth, and death has been implicated in mediating FA-induced modulation of gene expression (reviewed in^{3–6}). For instance, some members of the novel (n-) group of PKC isoenzymes that do not require Ca^{2+} but are sensitive to diacylglycerol (DAG) and to artificial PKC activators phorbol esters, have been reported to influence several genes including, for example, the LDL receptor gene, whose control is considered as the major mechanism by which dietary and hormonal factors control plasma lipid and cholesterol levels.^{7–11} Furthermore, PKCs have been implicated in mediating insulin action in a number of cell types and may contribute to the development of insulin resistance in tissues, including liver (reviewed in, e.g.,^{3,4}). Available data remain controversial indicating the existence of many gaps in our concepts of how PKCs function, however. In particular, liver cells have attracted less attention and are studied less extensively compared with adipocytes and skeletal muscles and while some have reported activation of PKC isoforms in hepato-

cytes or hepatoma cell lines,^{12–16} others have failed to obtain evidence of PKC activation in liver cells after insulin stimulation.^{17,18} Nevertheless, there seems to be a general consensus that abundance and excessive availability of plasma NEFA in obese individuals may lead to insulin resistance attempting to prevent accumulation of these energy substrates that may cause cell overload, lipotoxicity, or “lipid trauma” featuring metabolic syndrome.^{19,20} Indeed, more than a decade ago it has been suggested to view insulin resistance not solely as a disease of glucose homeostasis but, at least equally, as a lipid metabolism defect.²¹ Due to its anatomical location, the liver, supplied by blood through the portal vein is easily exposed to NEFA overflow released from visceral fat. Activation of PKC isoenzymes by ambient FA has been evidenced previously²² and interpreted as a result of the combined action of elevated intracellular long-chain acyl CoA (LCAC) derivatives that can give rise to diacylglycerol (DAG), a known potent activator of PKCs, accumulation of Ca^{2+} , and/or of NEFA themselves.^{22–24} Using lipid infusions in rats in vivo, Lam et al.²⁵ have identified the n-PKC- δ isoform as a potential important player in sustained insulin resistance induced by NEFA in this organ. At variance with this finding, Samuel et al.²⁶ have demonstrated abundance of the n-PKC- ϵ isoform, but not the PKC- δ isoform, in liver cell membranes from high-fat fed animals. Recent studies have also indicated that in obese subjects with type 2 diabetes, the activity of various PKC isoforms is higher than in controls.^{27,28}

In contrast to FA, several studies suggest that hormone dehydroepiandrosterone (DHEA), a C₁₉ steroid (3 β -hydroxy-5-androsten-17-one), and its sulfoconjugated derivative (DHEA-S) that are major secretory products of the human adrenal cortex²⁹ can protect against many age-associated diseases, including diabetes.^{30–32} Reportedly, DHEA stimulates insulin sensitivity in rat adipocytes, which has been attributed to activation of PKCs, phosphatidylinositol-3-kinase, and/or phospholipase D.^{33,34} DHEA has also been found to increase mRNA levels of peroxisome proliferator-activated receptors (PPARs) in adipocytes. Some of these effects can be mediated by one or more nuclear transcription factors.³⁵

An important position of small (~ 15 kDa) cytosolic, specific fatty-acid binding proteins (FABPs) that represent intracellular fatty acid carrier equivalents to plasma albumin has been evaluated recently (reviewed in³⁶). In particular, the L-FABP isoform is expressed in liver and at lower concentrations, also in kidney and intestine epithelium. The primary function of FABPs is to target fatty acids to their destinies in the cytoplasm and/or to nuclei in cells. Other functions of FABPs include protection of cells against the unfavourable effects of fatty acids. The expression of FABP is regulated primarily at the transcriptional level by a mechanism involving PPARs. Fatty acids can influence a variety of genes through their interaction with FABPs and, in turn, also modulate expression of FABP genes themselves.^{37–41}

During this pilot study, we utilized human hepatoma HepG2 cells that had been previously shown to be a reliable experimental model exhibiting lipoprotein metabolism, insulin sensitivity, responsiveness, and a multitude of lipid- and insulin-mediated effects.^{10,42} We focused on studying the mRNA level expression of PKC components of signalling cascades and FA-transporting machinery and questioned whether ambient NEFA would influence their mRNA levels in these cells when applied in low concentrations and prolonged time intervals (4 and 20 h). Furthermore, we attempted to evaluate effects of DHEA on the same components. We obtained data showing that NEFA were able to moderately up-regulate PKC- ϵ and - δ mRNA levels and, surprisingly, reduce L-FABP mRNA levels in HepG2 cells.

MATERIALS AND METHODS

Materials. HepG2 cells were obtained from ATCC (Rockville, MD, USA). The Protease Inhibitor Cocktail was from Roche Diagnostics (Mannheim, Germany). Dehydroepiandrosterone (DHEA), Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin, streptomycin, trypsin, the Phosphatase Inhibitor Cocktail 1, bovine serum albumin (BSA), fatty acid-free BSA, fatty acids (FA), phorbol myristate acetate (PMA), and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of the FA-BSA complex. FA-free BSA was dissolved in EMEM medium supplemented with 1% FBS and individual FA were added to this solution to achieve 2:1 FA/BSA ratios. The mixtures were vortexed and sonicated until optically clear and used immediately.

The cell culture. HepG2 cells were cultured in 75 cm² flasks with EMEM supplemented with 10% FBS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 IU/ml penicillin/streptomycin. The medium was refreshed every 2–3 days. The cells were maintained in a humidified 5% CO₂ and 95% air atmosphere at 37 °C to grow to about 80% confluence. The medium was then aspirated, the cells were washed twice with ice-cold phosphate buffered saline (PBS; 0.1 M phosphate buffer, pH 7.4, 0.9% NaCl), and switched to the same EMEM medium supplemented with 1% FBS and FA-BSA complexes added each to the final concentrations of 100 μ M FA. The final concentration of BSA was adjusted to 1% in all samples. The cells were then further incubated for 4 h or 20 hours before being fractionated and assayed. The same 1% FBS, 1% BSA medium without FA was used to incubate control cells. Incubation with 0.1 μ M PMA, a known PKC activator, in the medium was used as another control. In parallel incubations, DHEA was present at 1 μ M and 100 μ M concentrations. The data represent means of two separate analyses.

Quantitative mRNA analysis. Levels of mRNA were measured by a real-time PCR following reverse transcription (RT). Total RNA was isolated from about 80% confluent cell monolayers (10⁶ cells) using High Pure RNA Isolation kit and mRNA reversely transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics, Mannheim, Germany). Aliquots of the reversely transcribed samples were used for the LightCycler System using the FastStart DNA MasterPLUS SYBR Green I kit (Roche). Primer sequences were adopted from data of Berdiev et al.⁴³ (PKC- δ , PKC- ϵ), Blanquart et al.⁴⁴ (cyclophilin A), Kemp et al.⁴⁵ (glyceraldehyde-3-phosphate dehydrogenase; GAPDH), and Sumida et al.⁴⁶ (β -actin) or designed by us (L-FABP; 5'-GTGTCGGAAATCGTGC-3' and 5'-GATTATGTCGCCGTTGAG-3' are the forward and reverse primers, respectively) using LightCycler Probe Design software package (Roche) from gene sequences obtained from National Center for Biotechnology Information (NCBI) nucleotide database and synthesized by Metabion International (Martinsried, Germany). To compensate for variations in input RNA amounts, the mRNA values in each sample were normalized for an appropriate housekeeping gene (GAPDH, cyclophilin A, and β -actin for FA, and GAPDH and cyclophilin A for PMA). Controls without RT and with no cDNA template were tested in parallel with each assay and all samples were run in duplicate. The quality of RT-PCR products were further confirmed by melting curve analysis and by agarose gel electrophoresis of amplification products using visualization with ChemiGenius System (Syngene).

Fig. 1. Effects of saturated and monounsaturated fatty acids and PMA on the PKC- δ and - ϵ mRNA levels in HepG2 cells. (left), 4 h incubation period. (right), 20 h incubation period. PA, palmitic acid, ST, stearic acid, OL, oleic acid. Data represent means of two separate analyses.

Fig. 2. Effects of 4h and 20 h treatment by saturated and monounsaturated fatty acids and PMA on the L-FABP mRNA levels in HepG2 cells. PA, palmitic acid, ST, stearic acid, OL, oleic acid. Data represent means of two separate analyses.

Fig. 3. Effects of 20 h treatment by 100 μ M DHEA on the PKC- δ , - ϵ and L-FABP mRNA levels in HepG2 cells. Data represent means of two separate analyses.

RESULTS

HepG2 cells were incubated in the presence of palmitic, stearic, and oleic acids to evaluate whether the mRNA levels of intracellular FA metabolism regulators is influenced by ambient NEFA applied in low concentrations. After the incubation for 4 h and 20 h periods, quantitative real-time PCR was used to examine NEFA effects on PKC- δ and - ϵ mRNAs. In Figure 1 it can be seen that the saturated and monounsaturated acids modestly but rather selectively increased PKC- ϵ mRNA over the 4 h and/or 20 h incubation periods. The most potent stimulator was stearic acid that also slightly increased PKC- δ mRNA. The effect of oleic acid on PKC- ϵ mRNA was enhanced after 20 h while that of palmitic and stearic acids was no longer evident over the 20 h incubation period (Fig 1b). In comparison, artificial PKC activator PMA reduced (4 h) and then increased PKC- ϵ mRNA, and robustly increased PKC- δ mRNA levels over time (Fig 1a,b).

The mechanisms by which FA enter cells employ a variety of proteins.^{1,2} To address the question of whether FA can influence, at the mRNA level, metabolism of the proteins that mediate plasma membrane transport and target FA to different cytoplasmic destinations the effects of NEFA on mRNA expression of small cytosolic L-FABP protein that is known to play a crucial role in FA cytoplasmic trafficking were re-examined. Surprisingly, we found that palmitic, stearic, and oleic acids considerably reduced L-FABP mRNA levels in our model (Fig. 2). Additionally, PMA also reduced the mRNA level of this protein markedly (Fig. 2).

Protective action of DHEA against the adverse effects of NEFA has been reported repeatedly. Attempting to extend the knowledge of DHEA influence on its potential cytoplasmic targets, we examined the effects of physiological and supraphysiological concentrations (1 μ M and 100 μ M, respectively) on the abundance of PKC and L-FABP mRNAs in HepG2 cells. We found that the prolonged (20 h) treatment with 100 μ M DHEA concentrations increased PKC- δ mRNA and that of PKC- ϵ , in particular, while not changing L-FABP mRNA considerably (Fig. 3).

DISCUSSION

Previous studies have lent credence to the idea that PKCs, in addition to their role as physiological mediators of some of insulin effects, may be at least partially responsible for the development of insulin resistance. Previous authors have also reported that PKCs can be activated by ambient FA, both in vitro and in vivo conditions and in obese individuals (reviewed in^{23, 24}). Despite the well-described, acute effects of various agents on PKC activation occurring within minutes after the stimulation, it has been argued that FA-induced development of insulin resistance requires periods of hours, at least (reviewed in^{20, 47}). Indeed, prolonged treatments lasting for hours to days have been used to demonstrate effects of growth regulators and/or elevated plasma lipids on PKC translocation and insulin resistance development in vivo.^{25, 26} The long periods are assumed to be required to influence hepatic glucose production by impairing insulin control of gluconeogenesis and/or that of glycogenolysis, in particular, and to alter gene expression^{1, 10, 48} that can affect autoregulation in liver.^{20, 23, 47, 49} From this it is obvious that the prolonged, 4 h and 20 h incubation intervals as used in this work have been more appropriate in detecting sustained metabolic and/or adverse effects of FA, rather than those that might appear very early, i.e., within minutes, in cells after their stimulation. Of note, about 20 h in vitro incubation periods have been also reported as optimal to evaluate down-regulation of PKCs through the exposure of cells to phorbol esters.^{48, 50} However, it is clear from our experimental protocol that we did not address the question of whether the FA effects, observed by us, were direct or indirect ones. Potentially, some of the effects we observed could be those that persisted or were even delayed after the ambient FA had been consumed by the cells. In fact, the effects could be caused by FA themselves, by their metabolites (e.g., desaturation products, LCAC, DAG, lipoproteins, ceramides, products of FA oxidation, etc.), or by by-products of their metabolism, such as, e.g., reactive oxygen species. This important issue remains to be further explored.

Increase in mRNAs, which roughly coincide with at least partial down-regulation of their appropriate PKC protein isoforms following prolonged treatment of cells with insulin, have been reported by previous authors in non-liver cells.^{51, 52} Those authors hypothesized that such insulin-induced, plasma membrane receptor-mediated increase in PKC mRNAs following the redistribution and depletion of PKC isoenzymes is a compensatory mechanism to maintain PKC levels in insulin-stimulated cells. Although the activation of PKC isoenzymes by ambient FA has been evidenced previously at the protein level²², we have shown here for the first time that long-term treatment by FA can also lead to modulation of PKC mRNA levels, at least in HepG2 cells. Collectively, from our findings it may be surmised that studies on PKC dependency of cellular effects of ambient NEFA may not be sufficient to interpret within the limits of the current experimental paradigm that is predominantly focused on the PKC-iso-

form protein activation and/or translocation. Interestingly, as shown in figure 3, PKC mRNA levels have been also stimulated by prolonged (20 h) treatment with supraphysiological doses of DHEA.

While increasing PKC mRNAs, low ambient NEFA treatment yielded a decrease in mRNA of L-FABP in HepG2 cells. The literature reveals, however, that this finding is at variance with the results of other authors who clearly demonstrated FA to act as potent inducers of L-FABP gene expression in rodents.^{38–41} We do not believe that the finding is an artefact although the data that support this are only indirect, for the present: first, the finding has been further substantiated by the concordant effect of PMA (Fig. 2) and, second, it was not observed in the presence of any of the six polyunsaturated FA examined by us (including linoleic, arachidonic, phytanic, docosahexanoic, and two conjugated linoleic acids; data not shown). We assume this disparity is most probably the result of interspecies differences or a specific feature of HepG2 cells themselves. Alternatively, or additionally, the study design, composition of cultivation media and/or concentrations of FA should be considered as well. This problem deserves further attention. In this connection it is also relevant that FA entering hepatocytes are predominantly diverted to the re-esterification pathway, rather than oxidation, resulting in accumulation of DAG and triglycerides where they are stored before being secreted in VLDL, and/or to synthesis of ceramides (reviewed in^{23, 24}). L-FABP has been reported decreased in cells under conditions of limited oxidation of fatty acids.⁵³ Thus, the decrease in L-FABP mRNA expression as observed by us is consistent with the idea that it may reflect an adaptive mechanism aiming at to protect the cells against FA overflow. This is supported indirectly by the fact that knockout mouse lacking L-FABP gene or liver cells with reduced L-FABP expression exhibited both a reduced rate of long chain FA uptake and their oxidation under conditions of lipid supply.^{41, 54–56}

Overall, although there are various mechanisms described in the literature as to how NEFA may contribute to lipotoxicity, the present data direct attention to changes in mRNA levels of important intracellular signalling cascade components that may contribute to NEFA effects. Further analysis is clearly needed to provide conclusive evidence for the physiological importance of mechanisms underlying these findings.

ACKNOWLEDGEMENT

This work was supported, in parts, by the Ministry of Education, Youth, and Sports, Czech Republic, Grants COST B17.20, COST B17.001, and MSM 6198959205.

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