AS-30D hepatoma as a model to study on insulin resistance in vitro

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Studies on insulin resistance of liver cells are often performed with the use of various hepatoma cell lines. Such an approach allows investigating selected biochemical pathways at the cellular level. However, possible modifications of metabolic processes due to the neoplastic nature of such cells must be considered. Expanding the diversity of hepatoma cell lines used in metabolic studies could deliver new data for comparison with those obtained for other cell lines and should reduce the risk of misleading conclusions. In this study rat hepatoma AS-30D cells were tested as a potential model for studies on palmitate-induced insulin resistance. It was found that insulin-induced Akt kinase phosphorylation was substantially reduced in cells incubated with palmitate at a concentration as low as 75 μM. This effect was not accompanied by excessive reactive oxygen species (ROS) generation or increased Jun N-terminal kinase (JNK) phosphorylation. Moreover, preincubation of AS-30D cells with rosiglitazone, an antidiabetic agonist of peroxisome proliferator-activated receptor gamma (PPARγ), efficiently prevented the palmitate-induced insulin resistance. We conclude that AS-30D hepatoma cells may be used as a model sensitive to insulin and vulnerable to palmitate-induced insulin resistance.

Key words: AS-30D hepatoma, insulin resistance, palmitate, rosiglitazone

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INTRODUCTION

The recent dramatic increase of type 2 diabetes prevalence throughout the world makes it one of the most urgent medical problems of the 21st century. Sedentary life style, excessive calorific intake and improper chemical composition of highly processed food together with a favorable genetic background are the major factors increasing the risk of this disease (Ginter & Simko, 2012). Type 2 diabetes develops slowly and is preceded by pre-diabetic state of progressive insulin resistance of major tissues depending on this hormone: skeletal muscles, the adipose tissue, and the liver (Henriksen et al. 2011). Although the detailed biochemical mechanism of the decreased sensitivity to insulin is still obscure, it is commonly accepted that improper lipid metabolism, particularly in obese individuals, which leads to an excessive ectopic lipid accumulation in extra-adipose cells such as hepatocytes and muscle fibers, is of major importance (Snel et al., 2012). It results in numerous biochemical changes in those cells, including enhanced ROS generation, stimulation of stress response, and activation of protein kinases (e.g., unconventional PKC) which phosphorylate serine residues in the insulin-receptor substrate (IRS) protein preventing its phosphorylation at tyrosine residues, which is a prerequisite for insulin-induced signal transduction. Thus, one of the proposed anti-diabetic therapeutic strategies is to normalize the overall lipid metabolism.

Induction of insulin resistance in hepatic or muscle cells in vitro by incubation with palmitate is a commonly accepted and useful experimental approach to obtaining a well-defined model system for metabolic studies relevant to type 2 diabetes (Ruddock et al., 2008; Nakamura et al., 2009; Gao et al., 2010; Yang et al., 2012). The treatment results in intracellular lipid accumulation, resembling to some extent the consequences of dyslipidemia. Using cultured cells allows observing cell-specific metabolic abnormalities and effects of xenobiotics unhindered by the systemic response of the organism as a whole.

Basic biochemical studies of candidate pharmaceuticals for any diseases, including insulin resistance, need diverse biological models. Although animal experiments in vivo and on primary cell cultures derived from organs and tissues of choice are indispensable, preliminary experiments with stable cell lines are also desirable, at least for economical and ethical reasons. However, it is clear that the use of immortalized or neoplastic cell lines apart from obvious advantages may also increase the risk of misleading conclusions. Therefore, results of such experiments should be interpreted cautiously. To reduce the risk of cell-line-specific conclusions diverse cell lines originating from the organ of interest should be used and results obtained with the use of basically similar but independently established material should be compared critically to identify common tendencies. For most studies on hepatic insulin resistance only a few hepatoma cell lines (HepG2, H4IEC3 and Fao) have been used (Ruddock et al., 2008; Nakamura et al., 2009; Gao et al., 2010).

AS-30D rat hepatoma is poorly differentiated ascites tumor cell line, characterized by highly oxidative energy metabolism in the absence of glucose, which undergoes substantial glucose-dependent inhibition due to the Crabtree effect (Walborg et al., 1970; Rodriguez-Enriquez et al., 2001; Briscoe et al., 1994). The latter is a common property of rapidly proliferating cells including many other tumor cell lines. Intense glutamine consumption and utilization of ketone bodies as a source of acetyl-
CoA for elevated fatty acid and cholesterol synthesis are additional well described metabolic features of these cells (Briscoe et al., 1994; Holleran et al., 1995; Mathupala et al., 1995).

In this paper it is shown that hepatoma AS-30D strongly responds to insulin stimulation and this effect, detected by measuring of Akt kinase phosphorylation, is efficiently blocked when the cells are grown in the presence of palmitate. The obtained results indicate that AS-30D cells may be used as an experimental model of hepatic cells highly susceptible to palmitate-induced insulin resistance. Although the molecular mechanism underlying this feature has not been deciphered so far, it is noteworthy that an excessive ROS generation or phosphorylation of JNK protein kinase, which is a marker of cellular stress response, seem not to be necessary for the development of palmitate-induced insulin resistance in these cells.

MATERIAL AND METHODS

Preparation of palmitate-BSA complex. Palmitate-BSA complex was obtained according to the method described by (Cousin et al., 2001). Briefly, 100 mM sodium palmitate (Sigma Chemical Company, St. Louis, MO, USA) was prepared by incubation of appropriate amount of palmitic acid in 100 mM NaOH at 70°C for 30 min. Then one volume of sodium palmitate solution was added dropwise into three volumes of 10% BSA (Sigma-Aldrich Co. St. Louis MO, USA) heated to 50°C, agitated on a magnetic stirrer for the next 30 min, filtered through a 0.45 μm syringe filter and stored at minus 20°C. Prior to use the solution was heated at 50°C for 15 min. The actual concentration of palmitate in the complex was determined using a Nefa assay kit (Randox).

Cells culture and treatment. AS-30D hepatoma cells kindly provided by Dr. Antonio Villalobos (Institute for Biomedical Research, National Research Council and Autonomous University of Madrid, Spain) were grown in RPMI 1640 medium (The Institute of Immunology and Experimental Therapy, Wroclaw, Poland) supplemented with 2 mM glutamine, 10% Fetal Bovine Serum (Gibco Life Technologies, Invitrogen) and gentamycin (25 μg/ml) in a humified atmosphere of 5% CO₂/95% air at 37°C. Cells were subcultured every two days, at the density ranging between 180000 and 200000 cells per ml. 1 μM Rosiglitazone (from 1 mM stock dissolved in DMSO) or DMSO at the same final concentration 0.1% (control cells) were added to the cell suspension 72 h prior to the experiment. Insulin resistance was induced by incubation of cells with palmitate for 18 h prior to the experiment. Insulin (10 nM) dissolved in the incubation medium was added for the last 20 minutes of cell incubation.

Preparation of cell lysates and Western blotting. Samples of cell suspension (approx. 5×10⁶ cells) in the growth medium were centrifuged and pellets were rinsed twice with cold (4°C) PBS. Then, 300 μl of ice-cold lysis buffer (Cell Signalling Technology) supplemented with protease inhibitors cocktail (Roche), NaF (10 mM) and PMSF (1 mM) was added to each sample and the suspension was forced through a thin needle 10 times. The lysates were incubated for 20 minutes on ice and centrifuged at 15000×g at 4°C for 20 minutes. Supernatants were transferred into fresh tubes. Protein concentration was measured according to the Bradford method using the Protein Bio-Assay Kit (Bio-Rad) (Bradford, 1976). Then one volume of sample buffer was added to three volumes of each supernatant and the samples were boiled at 95°C for 5 minutes. After cooling, they were stored at −20°C. Polyacrylamide gel electrophoresis (PAGE) under denaturing conditions in the presence of 0.1% sodium dodecyl sulphate (SDS) was performed and then proteins were transferred onto nitrocellulose membranes. To detect selected proteins specific primary antibodies were used: UCP2 (Abcam), Akt, phospho-Akt, JNK, phospho-JNK (Cell Signalling), PPARγ (Santa Cruz Biotechnology). All secondary antibodies conjugated with horseradish peroxidase (HRP) were obtained from Abcam. Chemiluminescent substrate Luminata Classic or Crescendo (Millipore) were used for HRP detection. The intensity of bands corresponding to selected proteins was calculated densitometrically and expressed in a relation to intensity of band corresponding to β-actin (Monoclonal Anti-β-Actin–Peroxidase antibody, Sigma).

ROS measurement. Reactive Oxygen Species were estimated with the DFFH2-DA fluorescent probe (Molecular Probes, Eugene OR, USA). Samples of 2×10⁶ cells suspended in growth medium were centrifuged and pellets were rinsed twice with cold (4°C) PBS, suspended in 1 ml of the same buffer containing 20 μM probe and incubated for 30 min at 37°C. Fluorescence was measured at 520 nm with excitation at 485 nM using flow cytometry (FACS Calibur, Becton-Dickinson).

Lipid extraction and TLC. Lipids were double-extracted according to the method of Bligh & Dyer (1959). Neutral lipids were separated by TLC on silica gel (Merck) in the solvent system containing hexane/isopropyl ether:acetic acid (60:40:3). To visualize separated lipids, the plates were immersed in 10% CuSO₄ in 8% phosphoric acid and heated at 140°C for 20 min. The proportion of individual lipids was estimated on the basis of spot intensity analyzed densitometrically with the use of a Bioimager (Syngen, Ingenius). Total cellular triacylglycerol content was estimated colorimetrically with the use of the enzymatic Triglyceride Quantification Kit (Abcam).

Oil-red lipid staining. Oil-red (Sigma-Aldrich Co., St. Louis MO, USA) (0.5 g) was suspended in 100 ml of isopropanol, shaken overnight and filtered through a double Whatman filter. Immediately before use it was mixed with 0.66 volumes of water. A sample of cell suspension containing 5×10⁶ cells was centrifuged (1000 × g for 3 min) and rinsed twice with PBS. After careful removing of the supernatant the cellular pellet was fixed with 10% paraformaldehyde in PBS, incubated for 30 min at room temperature, centrifuged and rinsed twice as previously. Final pellet was suspended in the Oil-red solution, incubated for 1 h at room temperature, centrifuged and rinsed with PBS as previously. Stained cells were observed under a light microscope.

Statistical analysis. Data shown are means ± S.D. for the number of separate experiments indicated in the figure legends. The statistical significance of differences was calculated using Student's t-test.

RESULTS AND DISCUSSION

Palmitate decreases insulin-induced Akt phosphorylation

Incubation of diverse cells, including primary hepatocytes, hepatoma, primary muscle fibers and myotubes derived from immortalized C2C12 cells, with palmitate results in substantial insulin resistance manifested as a
reduced Akt phosphorylation upon treatment with insulin (Mordier & Iynedjian, 2007; Ruddock et al., 2008; Fen et al., 2012). This phenomenon resembles to some extent the in vivo insulin resistance of liver and muscle tissue observed in humans suffering from hyperlipidemia and/or type 2 diabetes, in experimental animals with induced obesity and dyslipidemia, and in genetically modified animals with aberrant fatty acid metabolism. It is also used to assess the cellular susceptibility to insulin-induced metabolic response. As shown in Fig. 1, incubation of AS-30D hepatoma cells with palmitate results in a dose-dependent decrease of insulin-induced Akt phosphorylation. Since an exposition of the cells to 50 µM palmitate resulted in a reduction of Akt phosphorylation by 50% while, 100 µM palmitate almost completely abrogated the insulin sensitivity, 75 µM palmitate was chosen for further studies. The resulting, highly reproducible and substantial but incomplete reduction of insulin-induced Akt phosphorylation in cells exposed to 75 µM palmitate (see Fig. 1) allows observing not only an increase but also a potential further reduction of the insulin sensitivity upon additional cell treatment. Thus, the natural sensitivity to insulin and the palmitate-induced insulin resistance of the AS-30D hepatoma indicate that these cells could be used as a model in studies focused on insulin-evoked responses of liver-derived cells. It also seems that the palmitate concentrations sufficient to induce substantial or complete insulin resistance are profoundly lower than those needed for a comparable response in other lines of hepatoma cells studied to date. For the Fao hepatoma cells an effective concentration of palmitate was described to be above 100 µM, but a significant decrease in Akt phosphorylation upon treatment of those cells with insulin was observed in the presence of 250 µM palmitate (Ruddock et al. 2008). Similarly, an evident decrease (by 40% and 60% of control in HepG2 and H4IIEC3 cells, respectively) of the phospho-Akt/ Akt ratio (the measure of insulin-induced response) was observed after preincubation of these cells with 250 µM palmitate (Nakamura et al., 2009; Gao et al., 2010). However, quantitative comparison of results obtained for different cell types and delivered by various laboratories are difficult and may be unconvincing, particularly if there are only very few data available. Nevertheless surprisingly high susceptibility of AS-30D cells to palmitate in term of induction of insulin resistance is noticeable. It may reflect special metabolic properties of these cells but it cannot be excluded that it results from different cell culturing conditions and higher availability of palmitate than in a case of other hepatoma cells. The latter may depend on whether the cells are growing in suspension (AS-30D hepatoma) or as an adherent monolayer (FAO, HepG2, H4IIEC3). Moreover, different methods of palmitate-BSA complex preparation may result in not the same response of cells to this fatty acid.

**Accumulation of glycerolipids, oxidative stress and JNK phosphorylation**

A reduced responsiveness of palmitate-treated hepatic cells to insulin was previously found to be accompanied by increased ectopic accumulation of acylglycerols, an excessive formation of reactive oxygen species and enhanced cellular stress response (Lee et al., 2010). These changes were suggested to be an important cause of insulin resistance. However, in contrast to data described by Gao (2010), an incubation of AS-30D hepatoma cells with palmitate at a concentration fully efficient for induction of insulin-resistance (75 µM) does not induce massive lipid accumulation, while 600 µM palmitate increases intracellular total acylglycerol content indicating a potential ability of these cells to acylglycerol storage (Fig. 2A). However, more precise thin-layer chromatography data analysis has revealed that an exposition of these cells to 75 µM palmitate for 18 h results in a moderately increased in diacylglycerol (DAG) content, without any significant influence on the intracellular triacylglycerols (TAG) (Fig. 2B–C). A similar effect was previously observed by Lee and co-workers in Hep-G2 cells (Lee et al., 2010), although the increase of the DAG level upon treatment with palmitate was more pronounced than that shown here. However in their experiments palmitate was applied at 0.5 mM concentration, thus claimed as decreasing cells viability. Other authors found substantially increased intracellular lipid accumulation in Hep2G.
cells exposed to 108 mM palmitate for 24 h (Wu et al., 2007), but usually 250 mM palmitate is used to induced insulin resistance in these cells (Gao et al., 2010). Also Oil-red staining of AS-30D cells did not indicate any visible increase in intracellular lipids unless the palmitate concentration was much higher than that required for insulin resistance to be induced (Fig. 2D). This observation underlines the striking difference between AS-30D and other hepatoma cells in terms of their susceptibility to palmitate-induced steatosis and insulin resistance, although some above-mentioned similarities particularly concerning absence of TAG accumulation in cells exposed to palmitate may be also found in the literature (Lee et al., 2010). In sum, while not excluding an excessive DAG accumulation as a cause of AS-30D hepatoma palmitate-induced insulin resistance our results indicate that the degree of cellular steatosis accompanying seriously reduced sensitivity to insulin seems to be much lower than in the case of other hepatoma cell lines.

An even more spectacular feature of AS-30D cells is a complete lack of an enhanced ROS generation (Fig. 3A) or JNK phosphorylation (Fig. 3B) upon treatment with 75 µM palmitate. In other hepatoma cells both these responses were proposed as important factors involved in the development of insulin resistance (Lee et al., 2010). The AS-30D cells are still able to produce ROS and exhibit a palmitate-induced stress response expressed as increased JNK phosphorylation (P-JNK), but again only in the presence of palmitate at much higher concentrations than those needed to induce insulin resistance (Fig. 3). While a quantitative comparison of cellular susceptibility to palmitate-induced insulin resistance of various lines of hepatoma may be difficult, the difference between AS-30D and other hepatomas in term of reactive oxygen species generation or JNK phosphorylation is of qualitative nature: palmitate applied at a concentration sufficient to induce sub-maximal insulin resistance in AS-30D cells does not stimulate ROS formation and JNK activation whereas in a case of other lines of hepatoma cause-effect relation between insulin resistance, enhanced reactive oxygen species and cellular stress response was suggested.

Thus data shown here allow separating insulin resistance from stress-related events and may suggest that treatment of cells with palmitate at high concentration, as described in other papers (Nakamura et al., 2009; Lee et al., 2010; Cao et al., 2012), may induce complex response which makes an interpretation of observed phenomenon more difficult. It other words, oxidative stress seems not to be necessary for the reduction of insulin sensitivity of AS-30D cells.

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**Figure 2. Effect of palmitate on acylglycerol accumulation in AS-30D cells**

Cells were incubated in the presence of albumin (control) or palmitate at concentrations as indicated, for 18 hours.

(A) Dose-dependent effect of palmitate on triacylglycerol accumulation (estimated with the use of Triglyceride Quantification Kit, Abcam). Data show mean values ± S.D. from three independent experiments. **p < 0.05. (B) Representative chromatogram of acylglycerols accumulated in AS-30D cells upon treatment with 75 µM or 300 µM palmitate. 1, control without palmitate; 2, 75 µM palmitate; 3, 300 mM palmitate. Identification of particular lipid band was based on the location of an appropriate standard and was performed for each chromatographic plate. Lane 4 derives from exactly the same chromatogram as other lanes shown in this figure. (C) Dose-dependent effect of palmitate on diacylglycerol accumulation estimated by densitometric analysis of thin-layer chromatograms. Data show mean values ± S.D. from three independent experiments. *p < 0.01. (D) Cells stained with Oil-Red. Data from one representative experiments.

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**Figure 3. Effect of palmitate on reactive oxygen species formation and JNK phosphorylation in AS-30D hepatoma cells**

(A) Reactive oxygen species generation was measured fluorimetrically with the DFFH2-DA probe using flow cytometry. Data show mean value of fluorescence ± S.D. for three independent experiments. **p < 0.05; *p < 0.01. (B) JNK phosphorylation estimated using Western blot analysis of cell lysate proteins. Results of one representative experiments out of three.
Figure 4. Effect of insulin and rosiglitazone on Akt phosphorylation in AS-30D cells treated with palmitate

(A) Western blot confirming presence of PPARγ in AS-30D cells.

(B) Response of AS-30D cells to insulin. Cells were preincubated with 1 µM rosiglitazone for 72 h and supplemented or not with 75 µM palmitate for the last 18 hours of this period. Data from seven independent experiments. *p < 0.01 for both palmitate plus insulin vs. control plus insulin, and for rosiglitazone plus palmitate plus insulin vs. palmitate plus insulin.

Effect of rosiglitazone. Available data indicate that antidiabetic drugs, thiazolidinediones (PPARγ agonists) may prevent/reverse insulin resistance of adipocytes, skeletal muscle and hepatocytes also in in vitro experiments (Kumar & Dey, 2003; Jiang et al., 2004; Wu et al., 2007; Burgermeister et al., 2006). Although PPARγ is the predominant isoform of PPAR in adipose tissue, it is also present in other cell types including hepatocytes (Guo et al., 2006; Rogue et al., 2010). Thus, PPARγ agonists could be expected to enhance the cellular response to insulin and affect hepatic metabolism. In fact, there is evidence from in vitro experiments indicating that thiazolidinediones (for example, pioglitazone or rosiglitazone) may modify lipid metabolism in liver cells (Djaouti et al., 2010; Chen et al., 2012).

Western blot result shown in Fig. 4A confirms the presence of PPARγ in AS-30D cells thus a rosiglitazone-induced improvement of insulin-sensitivity in these cells seems likely. As shown in Fig. 4B, preincubation with 1 µM rosiglitazone for 72 hours almost fully prevented the development of palmitate-induced insulin resistance in AS-30D cells. An increase of rosiglitazone concentration did not improve this effect, but at a lower concentration (100 nM) it was less effective (data not shown). Thus 1 µM concentration seems the most appropriate. This indicate that the sensitivity of AS-30D cells is similar to that described previously for Hep-G2 cells (Wu et al., 2007), although for some cells, such as adipocytes much higher concentrations of rosiglitazone were used to prevent their insulin resistance in vitro (Jiang et al., 2004). It cannot be excluded, however, that incubation of AS-30D cells with rosiglitazone for three days results in its intracellular accumulation and a consequent increase of the effective concentration. Importantly, incubation of hepatoma AS-30D cells with 75 µM palmitate with or without rosiglitazone did not affect their viability even when this drug was applied at a concentration as high as 100 µM. It was tested by flow cytometry analysis of living and fixed cells stained with propidium iodide. Results of such experiments firmly excluded both necrotic and apoptotic cell death (shown as supplementary data). The latter observation is important in view of data suggesting that rosiglitazone may be hepatotoxic in vitro (Guo et al., 2006). The lack of an effect of palmitate alone on the cell viability, indicates that their insulin resistance is not due to some non-specific palmitate-induced deterioration which could reduce cell sensitivity to insulin. In sum, we have presented here several interesting features of AS-30D cells, such as very high susceptibility to palmitate-induced insulin resistance, high sensitivity to rosiglitazone as a restorer insulin sensitivity, and high resistance to palmitate-plus-rosiglitazone-induced apoptosis. These characteristics allow studying effects related exclusively to decreased sensitivity to insulin, avoiding the cellular stress response potentially interfering with the response to insulin or antidiabetic effects of a drug tested.

In view of the data presented in this paper, the molecular mechanism of palmitate-dependent insulin resistance remains elusive. In principle it cannot be excluded that the small but detectable diacylglycerol accumulation, although much less remarkable than observed earlier in other hepatoma cells, is sufficient to reduce the insulin sensitivity. However, convincing explanation of this phenomenon as well as mechanism of rosiglitazone action in these cells need further studies.

CONCLUSIONS

AS-30D hepatoma responds to insulin stimulation by Akt phosphorylation, and this effect is reduced in a dose-dependent manner by preincubation with palmitate. A substantial desensitization of AS-30D cells to insulin is achieved at the presence of 75 µM palmitate, indicating their very high susceptibility. At the same time the cells show no symptoms of enhanced ROS generation, stress response, or decreased viability. These features distinguish them from other hepatoma cell lines commonly used in diabetes-related studies. The induced insulin resistance may be prevented by low doses of rosiglitazone applied two days prior to challenging the cells with palmitate. All these characteristics make the hepatoma AS-30D cells uniquely suitable for studies on insulin resistance. Additionally, their ability to grow in suspension may be advantageous in some experiments.

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REFERENCES


