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European Journal of Pharmacology 377 (1999) 241–252

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Metformin modulates insulin receptor signaling in normal and cholesterol-treated human hepatoma cells (HepG2)

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Received 15 October 1998; received in revised form 18 May 1999; accepted 21 May 1999

Abstract

The effects of the biguanide anti-hyperglycemic agent, metformin (*N,N′*-dimethyl-biguanide), on insulin signaling was studied in a human hepatoma cell line (HepG2). Cells were cultured in the absence (control cells) or in the presence of 100 μM of a cholesterol derivative, hemisuccinate of cholesterol. Cholesterol hemisuccinate-treatment alters cholesterol and lipid content of HepG2 and modulates membrane fluidity. Cholesterol hemisuccinate-treatment induces a decrease in insulin responsiveness and creates an "insulin-resistant" state in these cells. Exposure to 100 μM of metformin resulted in a significant enhancement of insulin-stimulated lipogenesis in control and cholesterol hemisuccinate-treated cells. In control cells, metformin altered glycogenesis in a biphasic manner. In cholesterol hemisuccinate-treated cells, metformin inhibited basal glycogenesis but restored insulin-stimulated glycogenesis. Hence, to understand the mechanism of metformin action, we analyzed early steps in the insulin signaling pathway, including insulin receptor autophosphorylation, mitogen-activated-protein kinase and phosphatidylinositol 3-kinase activities, in both control and cholesterol hemisuccinate-treated cells. Overall, the results suggest that metformin may interact with the insulin receptor and/or a component involved in the early steps of insulin signal transduction.

Keywords: Metformin; Cholesterol; Insulin receptor; Non-insulin dependent diabetes mellitus therapy

1. Introduction

Metformin is a powerful anti-hyperglycaemic agent, which is used in Europe for the treatment of diabetic patients with non-insulin dependent diabetes mellitus (Vigneri and Goldfine, 1987). Despite its wide utilization (Bailey, 1996), and the fact that many researchers have examined metformin effects on insulin response, the site(s) and mechanism(s) of metformin action remain unknown (Wiernsperger and Rapin, 1995). Metformin is a positively charged, hydrophilic, biguanide (Schäeffer, 1976a) that is non-permeant across plasma membranes. Thus, the first target of metformin may be at the plasma membrane, where it may interact with lipids (Schäeffer, 1976b) and proteins (Freisleben et al., 1992). The major effect of metformin is to either potentiate or mimic the actions of insulin (Rossetti et al., 1990; Wu et al., 1990). For instance, Stith et al. found that metformin stimulated insulin action by activation of the insulin receptor in oocytes (Stith et al., 1996). This group also observed an elevation of intracellular Ca^{2+} in response to metformin. Purello et al. suggested that metformin may influence cellular metabolism by potentiating certain insulin actions through mechanisms that may be beyond insulin receptor binding (Purello et al., 1988). Overall, the data suggest that metformin may act on certain insulin signaling pathways.

Insulin signal transduction pathways are well documented. Insulin binds to specific heterotetrameric membrane receptors ($\alpha_2\beta_2$) which possess an intracellular tyrosine kinase activity. Kinase activation after oligomerization of the receptors leads to substrate phosphorylation, e.g., insulin receptor substrate-1, and interactions with docking proteins via SH2/SH3 (*src* homology) or pleckstrin homology domains (Siddle, 1992; Cheatham and Kahn, 1995). Studies in which membrane lipid composition have been

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0014-2999/99/$ - see front matter
PII: S0014-2999(99)00386-6

modified have shown that lipids can affect insulin signaling (Spector and Yorek, 1985). Gould et al. (1982), Bruneau et al. (1987) and Leray et al. (1993) confirmed that tyrosine kinase activity could be modulated by the lipid environment of the receptor, both in vitro (reconstitution experiments) and in intact cells (lipid-treated cell lines). Membrane perturbations modify insulin biological effects to different extents (for review, Cremel et al., 1994; Meuillet et al., 1999). Understanding how the lipid environment modulates insulin receptor signaling will increase our knowledge of diabetes, one aspect of which is lipid metabolism disorders.

In this study, we used a cellular model in which insulin sensitivity was altered by supplementing the culture medium of HepG2 cells with a derivative of cholesterol, cholesterol hemisuccinate (Shinitzky, 1978; Meuillet et al., 1999). Using this model, metformin action was examined, on insulin binding, insulin receptor autophosphorylation, and on two insulin-dependent biological effects (glycogenesis and lipogenesis). The activity of phosphatidylinositol 3-kinase and mitogen-activated-protein kinase, two enzymes involved in well characterized insulin transduction pathways, were also studied in the presence of metformin in control and cholesterol hemisuccinate-treated cells.

2. Materials and methods

2.1. Cell culture

Human hepatocarcinoma HepG2 cells were obtained from ATCC (Rockville, MD, USA). Cells were grown in 75 cm^2 plastic tissue culture flasks or in multi-well plates for all experiments, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco, Grand Island, USA) and containing 4.5 g/l glucose, 100 U/ml penicillin and 100 μg/ml streptomycin under a 5% CO_2 atmosphere. In order to change the lipid composition of their cell membranes, HepG2 cells were grown for 2 to 4 passages in culture-medium supplemented with 100 μM cholesterol hemisuccinate (Sigma) as described previously (Meuillet et al., 1999). Beyond 100 μM, cholesterol hemisuccinate was toxic to the cells as shown by lactate deshydrogenase release tests. All experiments were performed near the exponential phase of cell growth. Results of all experiments were normalized according to protein content of cell extracts.

2.2. Metformin pretreatment

Metformin was obtained from Lipha Pharmaceuticals (Lyon, France). Metformin action on cell culture was tested by incubating the cells with various concentrations of metformin for 16 h. Control and cholesterol hemisuccinate-treated cells were incubated for 16 h with 100 μM metformin to approximate patient physiological conditions (780 mg 3 times a day) (Wiernsperger and Rapin, 1995).

2.3. Lipid analysis and membrane fluidity

For lipid analysis, confluent cell monolayers were rinsed 3 times in 9 g/l NaCl and resuspended in isobutanol. Lipids were extracted in chloroform/methanol according to (Leray et al., 1987). The cholesterol content was estimated by the colorimetric method using $FeCl_3$ and H_2SO_4 (Kates, 1972). For membrane fluidity measurements, cells were seeded into 60 mm dishes in Dulbecco's modified Eagle's medium with 10% fetal calf serum, grown to sub-confluence and transferred to Dulbecco's modified Eagle's medium with or without metformin (100 μM) for 16 h. Membrane fluidity was estimated by the fluorescence of two probes: diphenylhexatriene (DPH, 1 μM) and its cationic derivative the trimethylamonium diphenylhexatriene (TMA-DPH, 1 μM) as described by Bruneau et al. (1987). DPH is know to enter cells and label the endomembranes and lipid material inside intact cells (Johnson and Nicolau, 1977). Hence, DPH gives information about all membranes (plasma membranes and cellular membranes) whereas TMA-DPH is a more specific probe for study of plasma membrane fluidity in intact cells. Briefly, cells were washed 2 times in phosphate buffered saline at 25°C, gently scraped using a rubber policeman and resuspended at a concentration of 10^6 cells/ml. Membrane fluidity was estimated using an AMINCO SPF 500 equipped with polarization measurement accessories. Three dishes per treatment from three separate experiments were analyzed.

2.4. Cholesterol distribution

Filipin is an antifungic fluorescent molecule which complexes with cholesterol (Milhaud et al., 1989). To determine cholesterol distribution and verify its incorporation into the plasma membrane, cells were grown on glass coverslips in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Metformin was added as described previously. Coverslips were washed twice with phosphate buffered saline and immersed for 2 min in 0.5% glutaraldehyde and for 10 min in 1% glutaraldehyde. Monolayers were rinsed in Soerensen Buffer pH 6.9 (0.04 M NaH_2PO_4,H_2O, 0.06 M Na_2HPO_4,$2H_2O$), 3 times for 1 h. Coverslips were washed twice with phosphate buffered saline (pH 7.4) and then a 10 μM filipin solution was added, in the dark, for 5 min. Coverslips were washed 3 times with phosphate buffered saline for 10 min and mounted. Slides were visualized under a fluorescent microscope.

2.5. Insulin binding

Measurement of [^{125}I]iodotyrosyl^{A14} insulin (Amersham, Little Chalfont, UK) binding was carried out accord-

ing to previously described methods (Bruneau et al., 1987). Briefly, cells were incubated for 16 h in Dulbecco's modified Eagle's medium in the presence or in the absence of metformin. Monolayers were rinsed twice with phosphate buffered saline. Five hundred microliters binding buffer containing 100 mM HEPES pH 7.6, 150 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 10 mM glucose, 15 mM sodium acetate, 0.1% $CaCl_2$, 0.1% bovine serum albumin; 25 μl unlabeled insulin (bovine insulin was obtained from Novo, Copenhagen, DK), at different concentrations and 25 μl [^{125}I]insulin [20,000 to 40,000 cpm, specific activity: 74 Tbq/mmol; 200 Ci/mmol] were added to each well. The plates were then incubated at 16°C for 4 h. Cells were washed twice with Krebs Ringer Buffer (0.9% NaCl, 1.15% KCl, 2.1% KH_2PO_4, 3.8% $MgSO_4$, 0.2 M Na-pyruvate, 0.1 M Na-fumarate, 0.1 M Na-glutamate, 10 mM glucose, pH 7.4) and lysed in NaOH 0.2 N. Aliquots were removed for protein determination by the method of Bradford (1976) using bovine serum albumin as a standard. Radioactivity for the cell solute was determined in a gamma counter at 76% efficiency (LKB-Wallac 1260). All points were determined in duplicate for a minimum of three separate experiments.

2.6. Incorporation of [^{14}C]glucose into glycogen

Glycogen synthesis, estimated from the incorporation into glycogen of uniformly labeled D-[*U*-^{14}C]-glucose (Amersham), was performed as described by Staedel-Flaig and Beck (1978). Briefly, cells grown in 12 well plates, were incubated for 16 h in Ham F12 medium containing 1 g/l of glucose in the presence or in the absence of metformin. Stimulation, by indicated concentrations of insulin, for 2 h at room temperature was performed in phosphate buffered saline containing 0.1 g/l $CaCl_2$; 0.1 g/l $MgCl_2$; 1 g/l bovine serum albumin; 4 μCi/well D-[*U*-^{14}C]glucose (specific activity: 11 Gbq/mmol;295 mCi/mmol) and 5 mM glucose. Next, the wells were washed 3 times with ice cold Krebs Ringer buffer and 500 μl of 30% KOH was added to each well for 30 min at 37°C. Glycogen was precipitated by 10% Na_2SO_4/ethanol for 16 h at −20°C. Pellets, after centrifugation, were washed once in ice cold ethanol, solubilized in 500 μl H_2O and counted in a beta counter (LKB Wallac 1260). A total of six experiments, each in triplicate, were performed.

2.7. Incorporation of [^{14}C]acetate into lipids

Cells were deprived of serum for 16 h prior to this assay, done with or without metformin. HepG2 monolayers were incubated with [1.2-^{14}C]acetate (Amersham, 0.2 μCi/well; specific activity: 59 mCi/mmol; 2.18 GBq/mmol) in the presence of 200 μM cold acetate and insulin at the different concentrations (ranging from 0.01 to 10 nM) for 2 h at room temperature. After cell stimulation, plates were washed twice with ice cold phosphate buffered saline. Lipids were extracted by heating the plates for 1 h at 70°C with 2 ml of 5% saturated KOH in ethanol followed by the addition of 2.5 M H_2SO_4. After vigorous agitation with 3 ml of *n*-hexane, the organic layer was removed and counted in a liquid scintillation counter (LKB Wallac 1260). A total of four experiments, each in triplicate, were performed.

2.8. Detection of tyrosine phosphorylated proteins

Cells were seeded into 12 well-plates in Dulbecco's modified Eagle's medium with 10% fetal calf serum and transferred to Dulbecco's modified Eagle's medium with or without metformin for 16 h. Cells were then stimulated with 100 nM insulin at different times ranging from 0.5 to 5 min. The incubations were stopped by removing the medium, instantly freezing the cells in liquid nitrogen and extracting the proteins in a 1:1 mix of lysis buffer (20 mM Tris, 1% Triton X-100, 137 mM NaCl, 1 mM $NaVO_4$) and Laemmli sample buffer (Laemmli, 1970). Lysates were boiled for 5 min and analyzed by electrophoresis on 7.5% acrylamide gels. Proteins were then electrophoretically transferred to nitrocellulose membranes, pre-incubated in binding buffer (phosphate buffered saline with 3% bovine serum albumin) and incubated with anti-phosphotyrosine antibody (UBI, Lake Placid, USA, clone 4G10, 400 ng/ml). Immunoreactive bands were detected using the ECL system (Amersham) on FUJI RX film, and bands were quantified by densitometric scanning using a computer assisted analysis program (Scan Analysis, Biosoft).

2.9. Mitogen-activated protein kinase assay

Cells were treated as above. After stimulation, cells were lysed on ice in a lysis buffer as described by Meloche et al. (1992). Mitogen-activated protein kinase activity was measured using myelin basic protein (Sigma) as an exogenous substrate. Briefly, cells were scraped and lysates were clarified by centrifugation for 10 min at $10{,}000 \times g$. The supernatant was pre-cleared with protein A-Sepharose (Sigma). The supernatant was then immunoprecipitated with 2 μg anti-mitogen-activated protein kinase (UBI, rabbit polyclonal anti rat mitogen activated protein kinase R2) in the presence of protein A-sepharose. Pellets were washed 4 times in lysis buffer and once in kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol). Incubations were carried out in the same buffer containing 5 μCi [$\gamma-^{32}$P]-ATP (specific activity: 110 Tbq/mmol; 3000 Ci/mmol) and 0.25 mg/ml myelin basic protein. After 10 min, the reaction was stopped by the addition of 40 μl sample buffer, and aliquots were electrophoresed on 5–15% sodium dodecyl-sulfate–polyacrylamide gel electrophoresis. The gels were subsequently exposed for autoradiography, and radioactivity of the ~21 kDa band was measured. A total of four experiments were performed.

2.10. Determination of phosphatidylinositol 3-kinase activity

HepG2 cells grown in 10 cm dishes, were serum starved overnight, with or without metformin. They were then stimulated with 100 nM insulin for 10 min, and washed twice with ice-cold phosphate buffered saline. Cells were lysed and harvested by scraping in 20 mM Tris HCl, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM VO_4Na, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride (Sigma) and 10 μg/ml leupeptin (Sigma) for 1 h with rolling agitation. Immunoprecipitation with antiphosphotyrosine antibodies and protein A-agarose was performed at 4°C, for 2 h. The pellet formed by the immune complex was washed once in phosphate buffered saline, once in 0.5 M LiCl, 0.1 M Tris HCl pH 7.5, and once in kinase buffer (20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 24 μM ATP). The phosphorylation of phosphatidyl inositol was determined by the addition of 0.8 mCi/ml [γ-^{32}P]ATP and 2 mg/ml phosphatidylinositol in kinase buffer. Lipid extraction was performed in chloroform:methanol (1/1). After thin layer chromatography and autoradiography, the spots corresponding to phosphorylated phosphatidylinositol were scrapped and counted. Data are presented for four independent experiments.

2.11. Statistics

Data were expressed as the mean ± S.E.D., data were analyzed by the unpaired Student's *t*-test. *P*-values below 0.05 were regarded as statistically significant.

3. Results

To understand the effects of metformin on control and cholesterol hemisuccinate-treated HepG2 cells, we analyzed its action at several levels of the insulin receptor pathway. Insulin receptor phosphorylation, insulin-stimulated phosphatidylinositol 3-kinase and mitogen-activated protein kinase activities and glycogenesis and lipogenesis were examined in control and cholesterol hemisuccinate-treated cells, in the absence or the presence of 100 μM of metformin.

3.1. Lipid composition and membrane fluidity in HepG2 cells

Changes in membrane composition and membrane fluidity have been described as an important feature of non-insulin-dependent diabetes (Tong et al., 1994). In order to mimic these changes which are characteristics for non-insulin-dependent diabetes, HepG2 cells were treated for 2 to 4 passages with cholesterol hemisuccinate as described previously (Meuillet et al., 1999). Lipid composition and membrane fluidity of control and cholesterol hemisuccinate-treated cells were determined to verify that the cholesterol derivative was incorporated into the cells.

Cholesterol hemisuccinate induced a significant increase in cholesterol content in HepG2 cells (33.6 ± 3.5 μg/mg protein in control cells vs. 55.3 ± 4.7 μg/mg protein in cholesterol hemisuccinate-treated cells). Total lipid content was not modified (4.0 ± 0.07 vs. 3.93 ± 0.47 mg/mg protein for control and cholesterol hemisuccinate-treated cells, respectively.)

As the cholesterol composition was modified, membrane fluidity of intact cells, which also indicates cholesterol incorporation, was compared in control and cholesterol hemisuccinate-treated cells. Fluorescence anisotropy with two probes: TMA-DPH (1 μM) and DPH (1 μM) was performed on cell suspensions. DPH is incorporated into all membranes (plasma membranes and cellular membranes) whereas TMA-DPH only inserts into the outer leaflet of the plasma membrane (Johnson and Nicolau, 1977).

Results are summed up in Table 1. As expected, cholesterol hemisuccinate-treatment induced an increase in fluorescence anisotropy of both probes which corresponds to a decrease in membrane fluidity. As previously described by Muller et al. (1997), metformin had significant effects on membrane fluidity. In this study, 100 μM metformin increased membrane fluidity in control cells. At lower concentration (0.01 μM), metformin did not have a significant effect on membrane fluidity in control cells as measured by DPH and TMA-DPH in control cells. Surprisingly, 0.01 μM metformin increased membrane fluidity in cholesterol hemisuccinate-treated cells as measured by DPH and slightly with TMA-DPH.

Taken together, these results indicate that cholesterol hemisuccinate is incorporated into cell lipids, as evidenced by increased cholesterol levels and rigidification of cell membranes. Metformin has a slight effect on membrane fluidity suggesting a possible action at the surface of control and cholesterol hemisuccinate-treated cells.

3.2. Metformin effect on cholesterol redistribution in control and cholesterol hemisuccinate-treated cells

Recently, cholesterol has been suggested to play an important role in signal transduction (Pike and Miller, 1998). The modulation of cholesterol content can be critical for the activity of some enzymes. To localize cholesterol more precisely in the plasma membrane and to verify its incorporation at the plasma membrane level in cholesterol hemisuccinate-treated cells, we used an antifungic fluorescent molecule, filipin (10 μM). Filipin complexes with cholesterol (Milhaud et al., 1989). For control cells, we obtained uniform cell staining, whereas patches of cholesterol were observed in cholesterol hemisuccinate-treated cells (data not shown). In the presence of metformin, cellular morphology did not change for control or

Table 1
Metformin effect on membrane fluidity on control and cholesterol hemisuccinate-treated cells
Membrane fluidity was determined at 25°C on freshly scraped and suspended cells as fluorescence polarization of TMA-DPH (1 μM) and DPH (1 μM). Membrane fluidity was estimated using an AMINCO SPF 500 equipped with polarization measurement accessories.
Three dishes per treatment from three independent experiments were analyzed

Treatments	TMA-DPH			DPH		
	Control	Metformin (0.01 μM)	Metformin (100 μM)	Control	Metformin (0.01 μM)	Metformin (100 μM)
Control	0.224 ± 0.002	0.226 ± 0.001	0.216 ± 0.002[b]	0.123 ± 0.003	0.123 ± 0.003	0.125 ± 0.004
Cholesterol hemisuccinate	0.229 ± 0.002[a]	0.226 ± 0.002	0.230 ± 0.003[a]	0.145 ± 0.002[a]	0.139 ± 0.002[a,b]	0.145 ± 0.002[a]

[a] Values are significantly different ($P < 0.05$) between cholesterol hemisuccinate-treated cells and control cells.
[b] Values are significantly different ($P < 0.05$) between metformin-treatment and the corresponding controls.

cholesterol hemisuccinate-treated cells (data not shown). These results indicate that cholesterol hemisuccinate is incorporated in the plasma membrane of the cells and that metformin does not alter cholesterol distribution at the cell surface.

3.3. Effect of metformin on biological actions of insulin in control and cholesterol hemisuccinate-treated cells

Insulin is an anabolic hormone that stimulates glycogen and lipid synthesis. These biological actions were analyzed in HepG2 cells in the presence and absence of cholesterol hemisuccinate. The effects of metformin were studied on cholesterol hemisuccinate-treated and control cells.

3.3.1. Effect of metformin on glycogenesis in control and cholesterol hemisuccinate-treated HepG2

We studied insulin-stimulated glycogen synthesis in control and cholesterol hemisuccinate-treated cells (Fig. 1). In control cells, insulin induced a dose-dependent increase in glycogen synthesis with a maximal increase of approximately 1.4 at 10 nM insulin (Fig. 1A). cholesterol hemisuccinate-treated cells exhibited a different dose–response curve with a maximal increase of 1.15 at 0.01 nM insulin (Fig. 1B). Basal levels of glycogen synthesis (without insulin) were slightly higher in cholesterol hemisuccinate-treated cells as compared to control cells.

Next, the insulin effect on [^{14}C]glucose incorporation into glycogen was analyzed for different concentrations of metformin ranging from 0.01 to 100 μM. Increasing concentrations of metformin enhanced both basal and insulin-stimulated glycogen synthesis (data not shown), with 100 μM metformin showing the greatest increase.

Using different concentrations of insulin, we then examined insulin-stimulated glycogen synthesis in cells preincubated with 100 μM metformin. Metformin had strikingly different effects on control and cholesterol hemisuccinate-treated cells (Fig. 1A and B). Metformin increased glycogen synthesis in control cells as evidenced by a higher basal level (a 1.7-fold increase over non-metformin treated cells) and a shift to the left of the insulin concentration required for maximal insulin-dependent glycogen synthesis. One nanomolar of insulin was sufficient to obtain maximal insulin-stimulated glycogen synthesis (Fig. 1C). On the other hand, metformin decreased basal glycogen synthesis in cholesterol hemisuccinate-treated cells by a factor of 1.45-fold decrease as compared to non-metformin treated cells. In the presence of metformin, cholesterol hemisuccinate-treated cells exhibited maximal stimulation at a higher insulin concentration (10 nM) as compared to cholesterol hemisuccinate-treated cells in the absence of metformin (0.01 nM) (Fig. 1B).

Our observations on glycogen synthesis demonstrate a weak effect of insulin in these culture conditions in control and cholesterol hemisuccinate-treated cells. Nevertheless, we were able to detect a specific effect of metformin on insulin-stimulated glycogen synthesis in cholesterol hemisuccinate-treated cells. In order to clarify metformin effects on insulin-stimulated biological actions, we went on to study another metabolic process stimulated by insulin, lipid synthesis.

3.3.2. Effect of metformin on lipogenesis in control and cholesterol hemisuccinate-treated HepG2 cells

First, we investigated [^{14}C]acetate incorporation into control and cholesterol hemisuccinate-treated cells, in response to different insulin concentrations. Control and cholesterol hemisuccinate-treated cells exhibited similar dose–response curves with a maximal increase in lipid synthesis of approximately 1.6 to 1.7-fold over basal levels (Fig. 2A and B). Contrary to what was found for glycogen synthesis, cholesterol hemisuccinate-treatment significantly decreased lipid synthesis over control levels.

Next, lipogenesis was analyzed in control and cholesterol hemisuccinate-treated cells at different insulin concentrations, in the presence of 100 μM metformin. Metformin had a potent stimulatory effect on lipogenesis in control and cholesterol hemisuccinate-treated cells. Basal levels were slightly increased for both control and cholesterol hemisuccinate-treated cells. Insulin sensitivity was

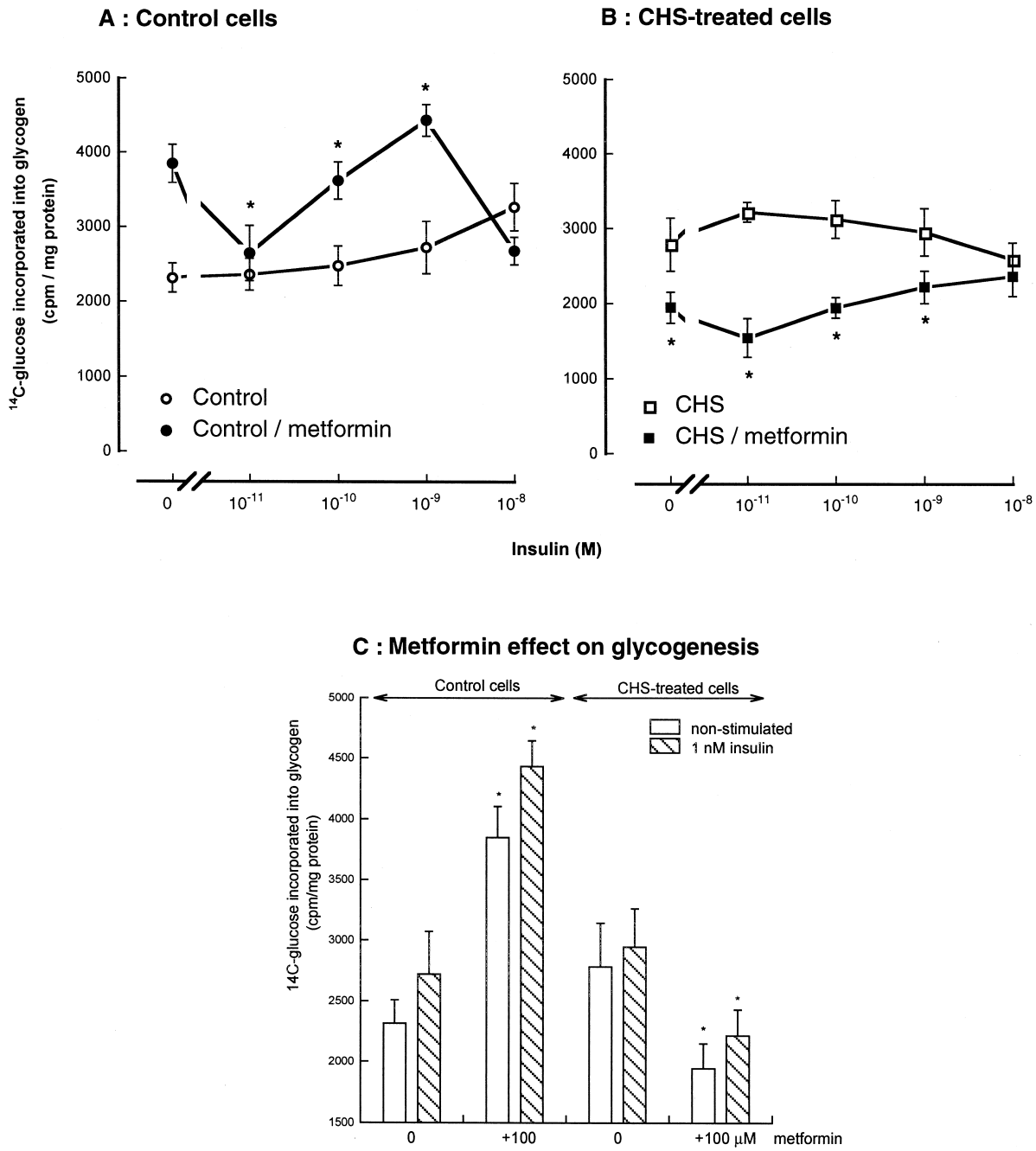

Fig. 1. Metformin effects on glycogen synthesis in control and cholesterol hemisuccinate-treated HepG2 cells. HepG2 cells (control (A); or cholesterol hemisuccinate-treated (B)) were stimulated for 2 h with the indicated concentrations of insulin, and the incorporation of [^{14}C]glucose into glycogen was measured in the presence (●, ■) or the absence (○, □) of 100 μM metformin as described in Section 2. Panel C of the figure summarizes the effects of metformin on glycogen synthesis in the absence (open bars) or in the presence (hatched bars) of 1 nM of insulin in control cells and cholesterol hemisuccinate-treated cells. All points were determined in triplicate, and represent the mean ± S.E.D. of six separate experiments. * Denotes significant differences ($P < 0.05$) between metformin-treatment and the corresponding control situation without metformin.

enhanced in control cells with a maximal effect seen at 0.1 nM insulin. In cholesterol hemisuccinate-treated cells, lipogenesis was significantly increased 2-fold at 1 nM insulin. In the presence of metformin, both curves (from control and cholesterol hemisuccinate-treated cells) were bell-shaped, which indicates higher sensitivity at lower insulin concentrations and a desensitization at higher insulin doses.

In summary, cholesterol hemisuccinate-treated cells exhibited a poor response to insulin for glycogen synthesis and a low lipid synthesis as compared to control cells. These results are in agreement with an "insulin-resistant"

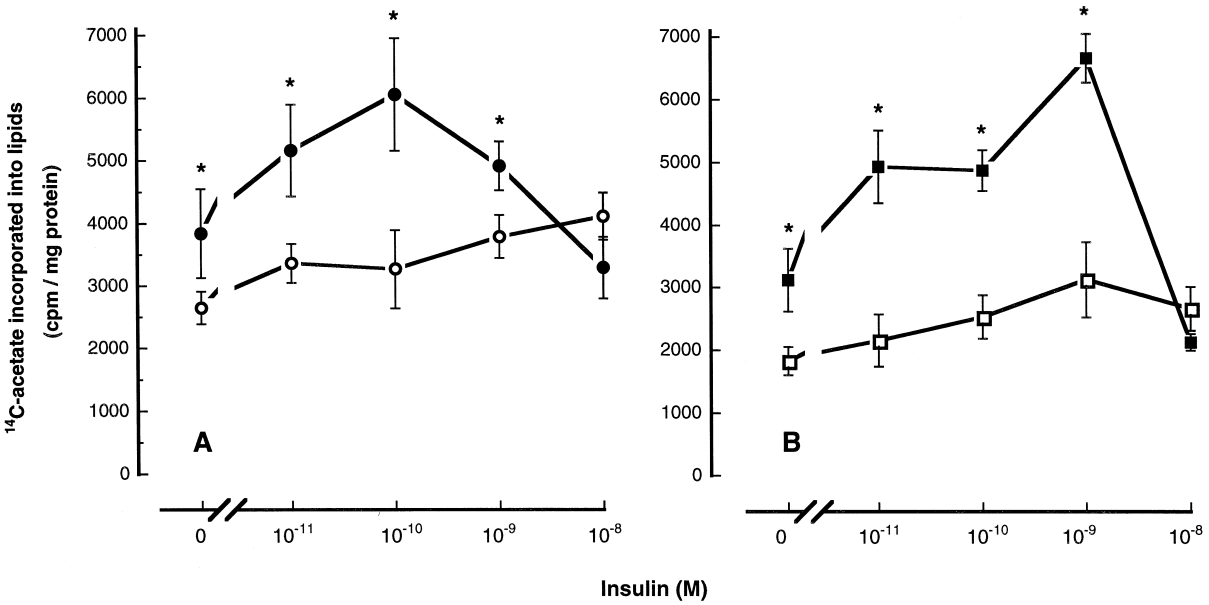

Fig. 2. Metformin effects on lipid synthesis in control and cholesterol hemisuccinate-treated HepG2 cells. HepG2 cells (control (A); or cholesterol hemisuccinate-treated (B)) were stimulated for 2 h with the indicated concentrations of insulin in the presence (●, ■) or absence (○, □) of 100 μM metformin, and the incorporation of [^{14}C]acetate into lipids was measured after extraction as described in Section 2. All points were determined in triplicate, and represent the mean ± S.E.D. of four separate experiments. * Denotes significant differences ($P < 0.05$) between metformin-treatment and the corresponding control situation without metformin.

state of the cells. Metformin slightly stimulated glycogenesis in cholesterol hemisuccinate-treated cells by restoring their ability to respond to increasing insulin doses. Metformin effects were more marked on lipogenesis which was increased in both control and cholesterol hemisuccinate-treated cells.

3.4. Effect of metformin on insulin receptor (binding and autophosphorylation), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase activities

Next, we investigated intermediary steps in insulin-induced signal transduction pathways. Insulin receptor autophosphorylation and insulin activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase were analyzed. These two enzyme are activated in response to insulin stimulation.

3.4.1. Lack of metformin effect on insulin binding

The first step in insulin action is the binding of insulin to its receptor. The potentiation of insulin action by metformin could result from an effect of the drug on insulin binding. To check this point, cells were pre-incubated for 16 h with 100 μM metformin and [^{125}I]insulin binding was determined for control and cholesterol hemisuccinate-treated cells as described previously (Meuillet et al., 1999). Competition binding curves were superimposable and Scatchard analysis did not reveal any major alteration in binding parameters (data not shown). Likewise, insulin receptor internalization measured as described by Roques et al. (1995) was not modified either by cholesterol hemisuccinate nor by metformin (data not shown).

3.4.2. Effect of metformin on insulin receptor autophosphorylation in control and cholesterol hemisuccinate-treated cells

Control and cholesterol hemisuccinate-treated cells were incubated with 100 nM insulin for different times ranging from 0.5 to 5 min. Tyrosine-phosphorylated proteins were visualized on Western blots with antiphosphotyrosine antibodies. Quantification of the bands detected on Western blots was performed using a densitometric scanner coupled to an image analysis system.

Insulin stimulated the phosphorylation of a major band at ~95 kDa, which was identified as the beta subunit of the insulin receptor, by immunoblotting and immunoprecipitation with anti-insulin receptor antibodies. Fig. 3 shows insulin receptor phosphorylation levels in control and cholesterol hemisuccinate-treated cells. Maximum phosphorylation of the receptor occurred after 5 min upon insulin stimulation in control cells. Kinetics of the phosphorylation of the insulin receptor were modified in cholesterol hemisuccinate-treated cells as compared to control cells. Basal phosphorylation (time 0) of the receptor was significantly higher in cholesterol hemisuccinate-treated cells than control cells ($P = 0.05$). After 30 s or 1 min of insulin stimulation, insulin receptor phosphorylation was similar in cholesterol hemisuccinate-treated and

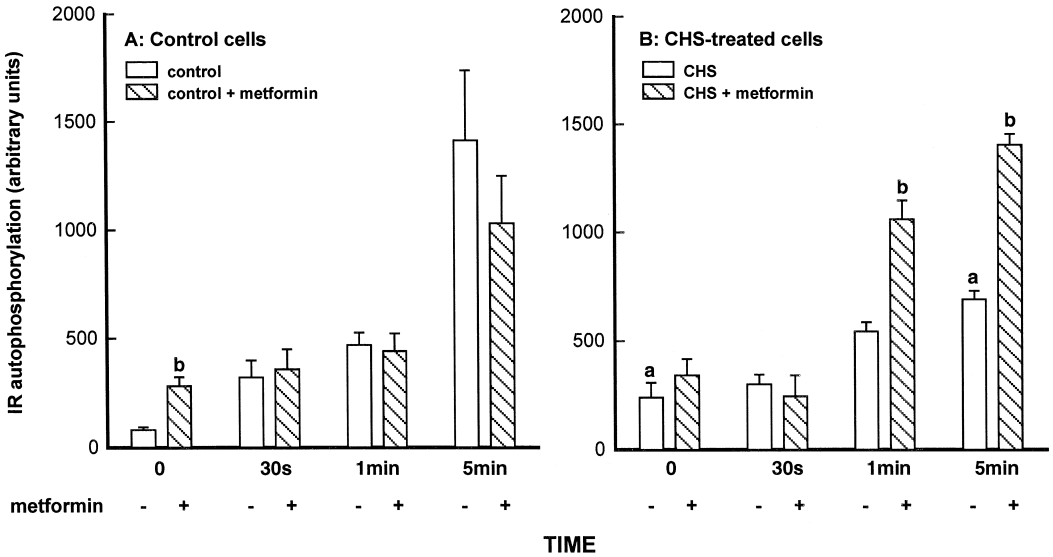

Fig. 3. Metformin effect on autophosphorylation of insulin receptors. HepG2 cells (control (A); or cholesterol hemisuccinate-treated (B)) were stimulated for the indicated times with 100 nM insulin, in the presence (hatched bars) or the absence (open bars) of 100 μM metformin. Cellular extracts were electrophoresed, transferred to nitrocellulose, and tyrosine-phosphorylated proteins were revealed with anti-phosphotyrosine antibodies using a peroxidase-coupled method as described in Section 2. A band at 95 kDa corresponding to the β subunit of the insulin receptor was quantitated after scanning. Results are depicted as the mean ± S.E.D. of four separate experiments. (a) Denotes significant differences ($P < 0.05$) between cholesterol hemisuccinate-treated cells and control cells; (b) denotes significant differences ($P < 0.05$) between metformin-treatment and the corresponding control situation without metformin.

control cells. However, after 5 min of insulin stimulation, insulin receptor was 50% less phosphorylated in cholesterol hemisuccinate-treated cells than in control cells ($P = 0.04$; Fig. 3, panel B, at 5 min, open bars compared to open bars panel A, same time). As already observed for glycogen and lipid synthesis, cholesterol hemisuccinate-treated cells behaved as an "insulin-resistant" model with decreased insulin receptor activity.

In the presence of 100 μM metformin (hatched bars, Fig. 3A and B), basal phosphorylation of the receptor was significantly increased in presence of metformin as compared to control cells without metformin. After 0.5, 1 and 5 min of insulin stimulation, insulin receptor autophosphorylation levels as quantified by densitometric scanning, were not altered as compared to controls. Overall, metformin did not affect insulin receptor phosphorylation kinetics in control cells. On the other hand, metformin affected insulin receptor autophosphorylation in cholesterol hemisuccinate-treated cells. At 1 and 5 min of insulin stimulation, metformin increased insulin receptor phosphorylation in these cells. Hence, metformin restored insulin receptor phosphorylation in cholesterol hemisuccinate-treated cells to control cell levels.

3.4.3. Effects of metformin on insulin stimulated pathways in control and cholesterol hemisuccinate-treated cells

In order to determine if a correlation existed between metformin effects on insulin biological actions and kinase activities involved in insulin transduction pathways, we analyzed metformin action on mitogen-activated protein and phosphatidylinositol 3-kinase activities in normal and cholesterol hemisuccinate-treated cells.

Mitogen-activated protein kinase activity was measured in control and cholesterol hemisuccinate-treated cells after 10 min of insulin stimulation (100 nM). Insulin significantly increased the incorporation of [^{32}P] into the protein myelin basic protein in control cells (Fig. 4). In these cells, insulin stimulated the incorporation of [^{32}P] by a factor of 2.2. The basal activity of mitogen-activated protein kinase in cholesterol hemisuccinate-treated cells was significantly lower as compared to control cells (left hatched bars). However, in these cells, insulin also significantly increased [^{32}P] incorporation, in this case by a factor of 2.7 over non-insulin treated cells.

Fig. 4 summarizes the observed effects of metformin on mitogen-activated protein kinase activity in the absence or presence of 100 nM insulin. Metformin did not affect mitogen-activated protein kinase activity (basal and insulin-stimulated kinase activity) in control cells. On the other hand, metformin increased basal and insulin-stimulated mitogen-activated protein kinase activity in cholesterol hemisuccinate-treated cells when compared to cholesterol hemisuccinate-treated cells without metformin. Mitogen-activated protein kinase activity in the presence of metformin was increased by a factor of 2-fold in basal cholesterol hemisuccinate-treated cells. In insulin-stimulated cholesterol hemisuccinate-treated cells, metformin increased mitogen-activated protein kinase activity by a

factor of 4.4 as compared to non-insulin stimulated cells (basal activity). Overall, metformin appeared to reverse the effects of cholesterol hemisuccinate-treatment on mitogen-activated protein kinase activity in these cells.

Phosphatidylinositol 3-kinase activity was measured under the same conditions as mitogen-activated protein kinase activity (Fig. 5). After 10 min, insulin induced a significant incorporation of [^{32}P] into phosphatidylinositol in control and cholesterol hemisuccinate-treated cells in the absence and in the presence of metformin. Due to the differences in basal activity of the enzyme in control and cholesterol hemisuccinate-treated cells, insulin increased phosphatidylinositol 3-kinase activity by a factor of 3.0 in control cells, but only by a factor of 1.4 increase in cholesterol hemisuccinate-treated cells. Basal activity of the enzyme was significantly higher in cholesterol hemisuccinate-treated cells as compared to control cells thus reducing the stimulation factor from 3.0 to 1.4. In the presence of metformin, basal activity of phosphatidylinositol 3-kinase was significantly increased in control cells as compared to the activity without metformin in these cells. When metformin was added to cholesterol hemisuccinate-treated cells, the drug produced a decrease in the effect brought about by cholesterol hemisuccinate alone, actually,

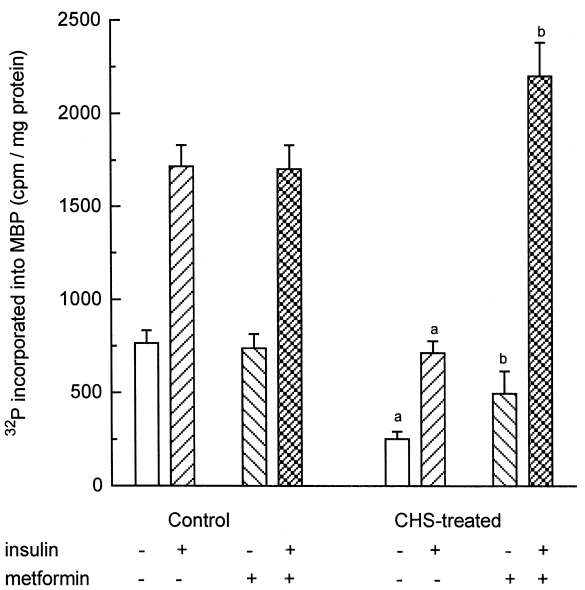

Fig. 4. Metformin effect on mitogen-activated protein kinase activity. HepG2 cells were stimulated for 10 min with 100 nM insulin, in the presence or absence of 100 μM metformin. Cellular extracts were immunoprecipitated with anti-mitogen-activated protein kinase antibodies and the pellet was assayed for phosphorylation of myelin basic protein as described in Section 2. After electrophoresis, a band at 21 kDa corresponding to the substrate was cut out and counted with a gamma counter. Results are depicted as the mean ± S.E.D. of four separate experiments. (a) Denotes significant differences ($P < 0.05$) between cholesterol hemisuccinate-treated cells and control cells; (b) denotes significant differences ($P < 0.05$) between metformin-treatment and the corresponding control situation without metformin.

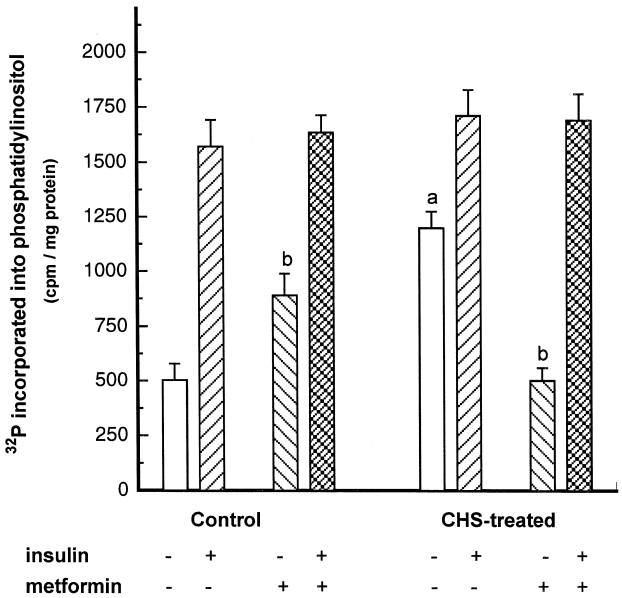

Fig. 5. Metformin effect on phosphatidylinositol 3-kinase activity. HepG2 cells were stimulated for 10 min with 100 nM insulin, in the presence or absence of 100 μM metformin as indicated. Cellular extracts were immunoprecipitated with anti-phosphotyrosine antibodies and the pellet was assayed for phosphorylation of phosphatidylinositol. After thin layer chromatography, the band corresponding to phosphorylated phosphatidylinositol was scraped and counted with a gamma counter. Results are depicted as the mean ± S.E.D. of four separate experiments. (a) Denotes significant differences ($P < 0.05$) between cholesterol hemisuccinate-treated cells and control cells; (b) denotes significant differences ($P < 0.05$) between metformin-treatment and the corresponding control situation without metformin.

appearing to reverse the effect of cholesterol hemisuccinate-treatment. Hence, upon insulin stimulation of cholesterol hemisuccinate-treated cells, phosphatidylinositol 3-kinase activity was comparable to that seen in control cells treated with insulin and metformin.

4. Discussion

Alterations in serum lipid fatty acids and cholesterol composition are common in diabetes, and are accompanied by modifications of membrane lipid composition and fluidity (Devynck, 1995). One way to alter membrane lipid composition and membrane fluidity is by cholesterol hemisuccinate-treatment. Taken together, the data from our recent previous study (Meuillet et al., 1999) and the data presented in this paper, cholesterol hemisuccinate-treatment induced changes in insulin responsiveness when compared to control cells. Biological actions, e.g., lipid synthesis and to a lesser extent, glycogen synthesis, and intermediary signaling pathways (such as phosphatidylinositol 3-kinase and mitogen-activated protein kinase) were significantly affected by this treatment. The effects of metformin used to treat patients with non-insulin-depen-

dent diabetes, was examined in control and cholesterol hemisuccinate-treated HepG2 cells. Metformin altered insulin sensitivity in cholesterol hemisuccinate-treated cells, as measured by lipid and glycogen synthesis. Insulin actions were differentially affected, with insulin-stimulated lipogenesis more sensitive than glycogenesis. Insulin binding was not affected by metformin in control or cholesterol hemisuccinate-treated cells. Metformin action was observed at early stages of the insulin intracellular signaling cascade, including insulin receptor autophosphorylation and mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities. Phosphorylation of the insulin receptor, which was altered in response to cholesterol hemisuccinate-treatment, was brought back toward control values by 100 μM metformin. Metformin increased basal phosphatidylinositol 3-kinase activity in control cells and decreased it in cholesterol hemisuccinate-treated cells. Mitogen-activated protein kinase activity, which was decreased by cholesterol hemisuccinate-treatment, was restored by 100 μM metformin. Overall, these results suggest that metformin can overcome insulin resistance induced by cholesterol hemisuccinate-treatment and that one site of action of metformin possibly resides within the insulin receptor and/or some component of the signaling complex associated with the insulin receptor.

Cholesterol hemisuccinate-treated cells were characterized by alterations in basal and insulin-stimulated levels of biological functions, such as lipogenesis and to a lesser extent glycogenesis, and by alterations in insulin responsiveness in the signaling pathways tested. A similar model of insulin resistance has been described using another hepatoma cell line, Zajdela Hepatoma Culture (ZHC cells). In ZHC cells, 25-hydroxycholesterol was found to induce a decrease in insulin responsiveness for amino-acid transport, glycogen synthesis and receptor autophosphorylation (Bruneau et al., 1987; Hubert et al., 1991). In the present study, analysis of cholesterol content and global membrane fluidity demonstrated that cholesterol hemisuccinate was incorporated into the plasma membrane. Cholesterol hemisuccinate-treatment affected glycogen and lipid synthesis. Moreover, insulin receptor phosphorylation was decreased in cholesterol hemisuccinate-treated cells as compared to control cells. This observation led us to conclude that cholesterol hemisuccinate-treatment induced an "insulin resistance state" in treated HepG2 cells. Mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities were also affected in cholesterol hemisuccinate-treated cells as compared to control cells. These two enzymes are activated by insulin and involved in signaling pathways downstream of ras (Pronk and Bos, 1994). Ras activity is modulated by its farnesylation (Hancock et al., 1989). As derivatives of cholesterol have been shown to act as negative regulators of hydroxymethylglutaryl-coenzyme A reductase activity and expression (Chambers and Ness, 1998), the decrease in mitogen-activated protein kinase activity induced by cholesterol hemisuccinate may be due in part to an inhibition of cholesterol biosynthesis, resulting in a decreased farnesylation of ras. Recently, Pike and Miller (1998) have demonstrated that depletion of cholesterol content is able to inhibit phosphatidylinositol turnover by delocalizing phosphatidylinositol bisphosphate in A431 cell lines. Taken together, cholesterol hemisuccinate mode of action may thus to be due to metabolic alterations and membrane fluidity changes (Shinitzky and Skornick, 1990) as well as local modifications of membrane lateral organization.

Metformin increased glycogen synthesis in control cells. This correlates with work from other laboratories (Purello et al., 1988; Melin et al., 1990), including the work of Lord et al. (1983) who showed an increase in glycogen synthesis in mouse soleus without an alteration in insulin binding. However, in our model, glycogen synthesis was minimally stimulated in response to insulin in control and cholesterol hemisuccinate-treated cells. It should be stressed that insulin stimulation of glucose incorporation was small, as is often the case for hepatoma cells. This may be due in part to the use of high glucose culture medium, required for maximal expression of insulin receptors in HepG2 cells (Biata et al., 1989). The fact that insulin action on glycogen synthesis is relatively small in HepG2 cells makes it difficult to precisely assess the importance of the changes observed in the presence of metformin. Hence, as insulin is also a potent stimulator of fatty acid synthesis and lipogenesis, metformin effects on this metabolic event were also examined. It was found that metformin greatly enhanced insulin stimulation of lipid synthesis in control and cholesterol hemisuccinate-treated cells. Melin et al. (1990) also reported a restoration of lipogenesis by metformin in rat insulin-resistant hepatocytes, but Anfosso et al. (1993) did not observe any effect of metformin on lipogenesis in HepG2 cells. This discrepancy may be due to shorter metformin incubation times used by the latter authors.

Since metformin altered insulin action, it was of interest to determine if it could modify insulin receptor binding, insulin receptor autophosphorylation, and subsequent intracellular steps. Metformin had no effect on competition binding curves, in agreement with several authors (Jacobs et al., 1986; Rossetti et al., 1990). To gain insight into the mechanism of metformin action, we analyzed tyrosine phosphorylation by western blotting with antiphosphotyrosine antibodies. We also analyzed mitogen-activated protein kinase and phosphatidylinositol 3-kinase activities after immunoprecipitation in control and cholesterol hemisuccinate-treated cells in the presence or absence of metformin. Metformin has been shown to exert several post-receptor effects, including an action on the receptor tyrosine kinase activity (Fantus and Brosseau, 1986; Stith et al., 1996). In cholesterol hemisuccinate-treated cells, where insulin receptor kinase activity was greatly diminished after 5 min, metformin restored insulin receptor autophosphorylation to levels similar to those observed in

control cells. Autophosphorylation of the insulin receptor also occurred at a faster rate in cholesterol hemisuccinate-treated cells in the presence of metformin (Fig. 3B, 1 min). Modifications in phosphorylation time-courses were also observed for mitogen-activated protein kinase and phosphatidyinositol 3-kinase activities, as well as for insulin receptor substrate-1 phosphorylation (data not shown). For both kinase activities (mitogen-activated protein kinase and phosphatidyinositol 3-kinase), the metformin effect was more pronounced in cholesterol hemisuccinate-treated cells where the drug was able to restore insulin responsiveness (stimulation factor were increased). It is noteworthy that metformin inhibited global glycogen synthesis in cholesterol hemisuccinate-treated cells as measured by [^{14}C]glucose incorporation, while it had a marked stimulatory effect on mitogen-activated protein kinase activity (Figs. 1 and 4). These results are consistent with the fact mitogen-activated protein kinase is not sufficient for regulation of most insulin metabolic actions (Denton and Tavare, 1995). Moreover, the observations on lipid synthesis also suggest that phosphatidylinositol 3-kinase alone may be insufficient to mediate metabolic and mitogenic effects of insulin (Krook et al., 1997). Overall, metformin was found to modify early events in insulin signaling at receptor and post-receptor steps.

In conclusion, metformin modulates biological effects in response to insulin and restores insulin responsiveness in cholesterol hemisuccinate-treated cells. Metformin effects are evidenced on early steps in the insulin signaling pathways. Overall, our data suggest that one possible mechanism of action of metformin on target cells is to enhance or modulate the responsiveness to insulin via a membrane-bound component, possibly associated with the insulin receptor.

Acknowledgements

This work was supported in part by a grant from Lipha, Lyon, France.

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