JCI The Journal of Clinical Investigation

Epidermal growth factor receptor defects in leprechaunism. A multiple growth factor-resistant syndrome.

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J Clin Invest. 1989;84(5):1569-1576. https://doi.org/10.1172/JCI114334.

Research Article

Leprechaunism is a rare genetic disorder characterized by severe growth retardation and insulin resistance. Maximal epidermal growth factor (EGF) binding was reduced in fibroblasts from three unrelated patients with leprechaunism (Ark-1, Can-1, and Minn-1) compared with control (0.8-2.2%/mg protein vs. 5.5%/mg protein). This was due to a decrease in receptor affinity in Ark-1 and Can-1 and a decrease in receptor number in Minn-1. In all cell lines, EGF-stimulated receptor autophosphorylation was also decreased to 18-60% of control, whereas EGF internalization and degradation was normal. Sphingosine (40 microM), a protein kinase C inhibitor, increased EGF receptor affinity twofold in control cells and six- to nine-fold in cells of leprechaunism. However, sphingosine did not enhance EGF-stimulated receptor autophosphorylation in either the controls or the patients' cells. By contrast, only one of the three cell lines of patients with the type A syndrome demonstrated a decrease in EGF binding and all demonstrated normal or near normal EGF-stimulated receptor autophosphorylation. These data indicate that in patients with leprechaunism, there are functional abnormalities of the EGF receptor, as well as of the insulin receptor, that may contribute to the severity of the syndrome. These data also suggest a role for the insulin receptor in maintaining normal EGF receptor function in these cells.

Epidermal Growth Factor Receptor Defects in Leprechaunism

A Multiple Growth Factor–resistant Syndrome

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Abstract

Leprechaunism is a rare genetic disorder characterized by severe growth retardation and insulin resistance. Maximal epidermal growth factor (EGF) binding was reduced in fibroblasts from three unrelated patients with leprechaunism (Ark-1, Can-1, and Minn-1) compared with control (0.8–2.2%/mg protein vs. 5.5%/mg protein). This was due to a decrease in receptor affinity in Ark-1 and Can-1 and a decrease in receptor number in Minn-1. In all cell lines, EGF-stimulated receptor autophosphorylation was also decreased to 18–60% of control, whereas EGF internalization and degradation was normal. Sphingosine (40 μM), a protein kinase C inhibitor, increased EGF receptor affinity twofold in control cells and six- to ninefold in cells of leprechaunism. However, sphingosine did not enhance EGF-stimulated receptor autophosphorylation in either the controls or the patients' cells. By contrast, only one of the three cell lines of patients with the type A syndrome demonstrated a decrease in EGF binding and all demonstrated normal or near normal EGF-stimulated receptor autophosphorylation. These data indicate that in patients with leprechaunism, there are functional abnormalities of the EGF receptor, as well as of the insulin receptor, that may contribute to the severity of the syndrome. These data also suggest a role for the insulin receptor in maintaining normal EGF receptor function in these cells.

Introduction

Leprechaunism is a rare genetic disorder characterized by elfinlike facies, intrauterine and neonatal growth retardation, reduced subcutaneous adipose tissue, acanthosis nigricans, genitomegaly, and death in infancy. Metabolic abnormalities include insulin resistance with severe hyperinsulinemia, postprandial hyperglycemia, and paradoxical fasting hypoglycemia (1, 2). We and others have shown defects in insulin binding and action in cultured, as well as circulating cells of patients with leprechaunism (2–7). Abnormalities in insulin receptor binding and in tyrosine kinase activity of partially purified insulin receptor preparations have been found (8, 9). However, the broad range of clinical findings and biochemical abnormalities suggest that alterations in other hormones and growth factors or their receptors could contribute to the pathogenesis of this disease.

Analogous to the insulin receptor, the epidermal growth factor (EGF)[1] receptor is a tyrosine kinase which undergoes ligand-stimulated autophosphorylation and acts as a phosphotransferase towards other proteins. The EGF receptor is a single polypeptide of ~ 170,000 D, having an extracellular EGF-binding domain, a single transmembrane domain, and an intracellular kinase domain. The tyrosine kinase activity of the receptor is thought to play a pivotal role in the biological effects of EGF. EGF binding also initiates rapid receptor-mediated endocytosis and subsequent ligand and receptor degradation in lysosomes, but the role of tyrosine phosphorylation in these processes is debatable (10, 11). EGF receptor function has been found to be altered by serine and threonine phosphorylation mediated by protein kinase C (12).

In this report, we have evaluated EGF binding, internalization and degradation as well as EGF receptor autophosphorylation in intact fibroblasts of leprechaunism. To examine the role of protein kinase C in any abnormalities, the effects of sphingosine, a potent reversible protein kinase C inhibitor (13), on insulin and EGF binding and EGF receptor phosphorylation were studied.

Methods

Materials. Na^{125}I-mouse EGF (153 μCi/μg) and [^{32}P]orthophosphate (3 Ci/mmol) were purchased from Amersham Corp. (Arlington Heights, IL) and New England Nuclear (Boston, MA), respectively. Mouse EGF was from Collaborative Research Inc. (Waltham, MA). D-Sphingosine was obtained from Sigma Chemical Co. (St. Louis, MO), and all other chemicals were of best available analytical grade. Tissue culture plasticware was from Nunc (Copenhagen, Denmark), Dulbecco's modified Eagle's medium (DME) and Earle's balanced salt solution (EBSS) from Gibco Laboratories (Grand Island, NY), and fetal calf serum (FCS) was supplied from Flow Laboratories, Inc. (McLean, VA).

Skin fibroblasts of Ark-1 were courtesy of Dr. J. Elders (Little Rock, AK); cells of Can-1 were the kind gift of the Department of Genetics, University of Manitoba (Winnipeg, Canada), and those of Minn-1 were obtained from the Mutant Cell Repository, Camden, NJ (catalogue no. GM5241). Cultured skin fibroblasts of patients with another insulin resistant condition, the type A syndrome were also studied. Fibroblasts of patients A-1, A-2, and A-3 (Boston) (14, 15) have been maintained in our laboratory. Control skin fibroblasts (n = 6) were obtained from normal volunteers and from American Type Tissue Culture, Rockville, MD (catalogue no. CRL1537, CRL1506, CRL1474, and CRL1477). All fibroblasts were maintained in DME with 10% FCS in humidified atmosphere containing 5% CO_2 at 37°C. Cells were maintained by subculturing 1:3 and used for experiments

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Received for publication 19 September 1988 and in revised form 28 June 1989.

J. Clin. Invest.

0021-9738/89/11/1569/08 $2.00
Volume 84, November 1989, 1569–1576

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; IGF, insulin-like growth factor; MSA, multiple stimulating activity.

2–3 d after reaching confluence; all cells were used between the 5th and 18th passages with little interpassage variation. All cells were fed at the same time and growth rates were similar.

EGF binding, internalization, and degradation. The fibroblasts were plated onto six-well (35-mm) dishes and were allowed to reach confluence. Before use, the cells were washed twice with chilled phosphate-buffered-saline (PBS) at pH 7.4 and once with EBSS containing 0.1% bovine serum albumin (BSA) and 25 mM Hepes at pH 7.4. The cells were then incubated in the same medium with ^{125}I-EGF (0.2 ng/ml) and various concentrations of unlabeled EGF (0–240 ng/ml) at 4°C. After 3 h the cells were washed twice with chilled PBS and solubilized with 0.5 ml of 0.1% sodium dodecyl sulfate (SDS). The cell lysates were counted in a gamma counter and protein concentration determined by the method of Bradford (Bio-Rad Laboratories, Richmond, CA). Specific binding was calculated as total minus nonspecific cell-associated radioactivity and all points were determined in duplicate. In some experiments, the cells were incubated with 40 μM sphingosine for 30 min at 37°C in the appropriate medium for binding. Sphingosine was present throughout the assay.

For internalization and degradation studies, steady-state EGF binding was achieved at 4°C and the cells were then placed in the incubator at 37°C for the indicated time to allow internalization of tracer. Cell surface bound EGF was removed by a 10-min acid wash (0.3 M acetic acid, 0.15 M NaCl, pH 3.0) at 4°C and the remaining cell-associated insulin was collected by solubilization of the cells with 0.1% SDS. The acid wash (surface bound tracer) and the SDS-lysates (internalized tracer) were counted in a gamma counter. This acid wash method removes 95% of cell-associated tracer when binding is performed at 4°C. Degradation of internalized tracer was determined by gel filtration chromatography on a Sephadex G-50 column as previously described (16).

EGF receptor autophosphorylation. Cells were plated onto 150-mm dishes and allowed to reach confluence. The cells were placed in serum-free DME with 0.1% BSA overnight and labeled for 2 h with phosphate-free DME containing [^{32}P]orthophosphate (0.4 mCi/ml). The labeled cells were stimulated with EGF (250 ng/ml) for 5 min after which the reaction was stopped instantaneously with liquid N_2, and the cells allowed to thaw into chilled solubilizing buffer containing 50 mM Hepes, 1% Triton X-100, 5 mM EDTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 2 mM sodium orthovanadate, aprotinin (1,000 kallikrein units/ml) and 2 mM phenylmethylsulfonyl fluoride at pH 7.4. The cell lysate was centrifuged at 200,000 *g* for 60 min and the supernatant was incubated with rabbit polyclonal anti-phosphotyrosine antibodies (17). There were ~ 4–4.5 × 10^6 cells per 150-mm disk and the solubilized extracts were normalized for protein content prior to immunoprecitation. The immune complexes were precipitated by Protein A (Pansorbin from Calbiochem-Behring Corp., San Diego, CA) and then prepared for SDS polyacrylamide gel electrophoresis under reducing conditions (100 mM dithiothreitol) according to the method of Laemmli (18). Autoradiography of the dried gels was performed and the appropriate areas of the radiographs were quantitated by scanning densitometry. An important aspect of this report is the use of anti-phosphotyrosine antibodies to immunoprecipitate the EGF receptor as well as potential substrate phosphoproteins. The amount of phosphorylation of the EGF receptor was expressed as the percentage of control maximum after subtracting the basal value from each stimulated value. In some phosphorylation experiments, cells were incubated with 40 μM sphingosine for 30 min at 37°C before addition of EGF.

Results

EGF binding. EGF binding was performed on intact cells at 4°C for 3 h and the specific ^{125}I-EGF binding per milligram of protein was plotted vs. the concentration of EGF. Control fibroblasts had a mean specific binding of 5.5%/mg of protein with the range of 3.0–12.6%/mg. EGF binding in the three cell lines of patients with leprechaunism was consistently lower than that of control cells (Figs. 1 and 2). At tracer concentrations, the percent EGF bound/mg protein was 1% for cells of Can-1, 2.2% for cells of Ark-1, and 0.8% for the cells of Minn-1. Thus, compared with the mean control EGF binding, the leprechaun cell lines demonstrated a 60–85% reduction in maximal EGF binding.

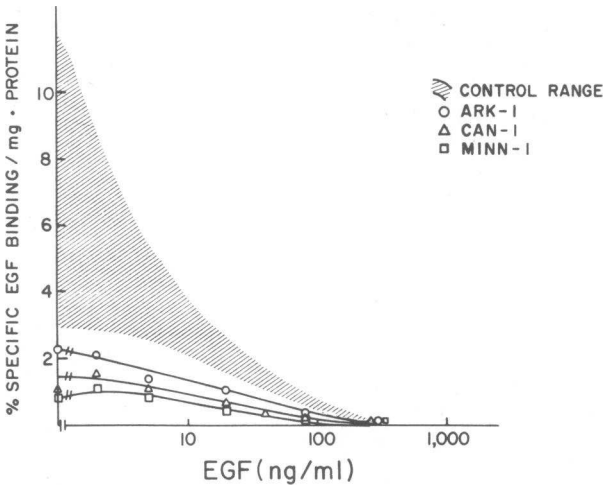

Figure 1. Specific ^{125}I-EGF binding to fibroblasts of patients with leprechaunism. Confluent monolayers of fibroblasts from controls and patients with leprechaunism (Ark-1, Can-1, and Minn-1) were incubated with ^{125}I-EGF (0.2 ng/ml) and various amounts of unlabeled EGF (0–240 ng/ml) at 4°C for 3 h. Unbound EGF was removed as described in Methods and the cell-associated counts were determined. Nonspecific EGF binding was < 0.1% of total tracer and was not different between patient and control cell lines. The points represent the means of four experiments. The shaded region denotes the absolute control range.

The EGF concentration required for half-maximal inhibition of tracer binding (ED_{50}) was 7–12 ng/ml in control cells. In cells of Ark-1 and Can-1, the ED_{50} was 30 ng/ml and in the cells of Minn-1, it was 15–20 ng/ml. Scatchard analysis revealed normal cells to have ~ 3.2 × 10^4 receptors per cell while cells of Ark-1 and Can-1 had 2.9 and 2.3 × 10^4 receptors per cell respectively and cells of Minn-1 had only 1.0 × 10^4 sites per cell. The Scatchard plots yielded single straight lines in each case, consistent with a single class of binding sites. The calculated affinity constants (K_d [nanomolar]) were 3.2, 2.9,

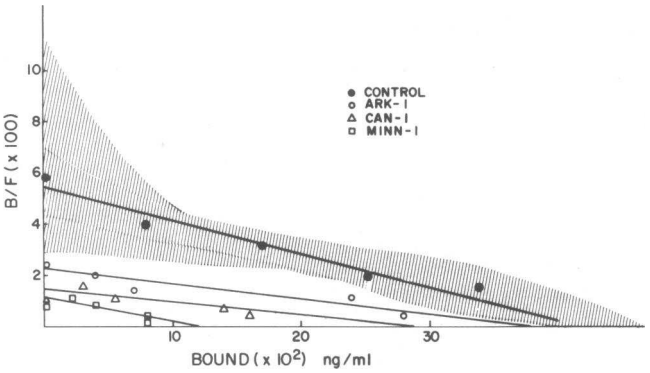

Figure 2. Scatchard analyses of EGF binding in cells of leprechaunism. The data from Fig. 1 are replotted as bound/free (*B/F*) of EGF as a function of bound EGF.

2.3, and 1.0 in control cells and cells of Ark-1, Can-1, and Minn-1, respectively.

For comparison, EGF binding was examined in the fibroblasts of patients with type A syndrome (A-1, A-2, and A-3 [Boston]), another syndrome of severe insulin resistance with altered insulin receptor function (Figs. 3 and 4). Previous studies had revealed that cells of A-1 and A-2 had reduced insulin binding, whereas cells of A-3 had normal insulin binding but reduced insulin receptor kinase activity. Two cell lines (A-1 and A-3) had maximal EGF binding within the upper part of the normal range (10.4%/mg and 9.7%/mg, respectively), whereas the cells of A-2 had 20% of mean control value. Marginally increased receptor affinity with an ED_{50} of 3 ng/ml was observed in the cells of A-1 and A-3 and a reduced receptor number of 1.0×10^4 per cell in cells of A-2.

EGF internalization and degradation. EGF internalization and degradation were examined at 37°C after steady state binding was achieved at 4°C. Internalization was normalized for specific binding and per cent internalized tracer was plotted vs. time (Fig. 5). Internalization was linear over the 25 min at 37°C. Of cell-associated tracer, 50% was internalized within 15 min in the control cell lines. Internalization in the cells of the patients with leprechaunism was within the normal range and followed a similar linear course.

To assess the amount of degradation of internalized tracer, internalization was allowed to proceed for 20 min after which cells were subjected to an acid wash to remove surface-bound EGF and solubilized. EGF degradation in the cell lysates was assessed by gel filtration. At this time point, ~ 65% of tracer was internalized in all cell lines studied. The percent EGF degradation in the cells of Ark-1, Can-1, and Minn-1 was 16%, 23%, and 21%, respectively, with normal range being 15–22%. Thus, both internalization and degradation of tracer were not different from controls.

EGF receptor autophosphorylation. EGF-stimulated tyrosine phosphorylation was examined using intact cells labeled with ^{32}P and a polyclonal anti-phosphotyrosine antibody (Fig. 6). Since a 5-min stimulation with EGF resulted in linear kinetics of receptor autophosphorylation, this point was chosen for comparison of the different cell lines (19). EGF-stimulated receptor phosphorylation, represented by a 170–180-kD band on SDS gels of the immunoprecipitates, was decreased in all

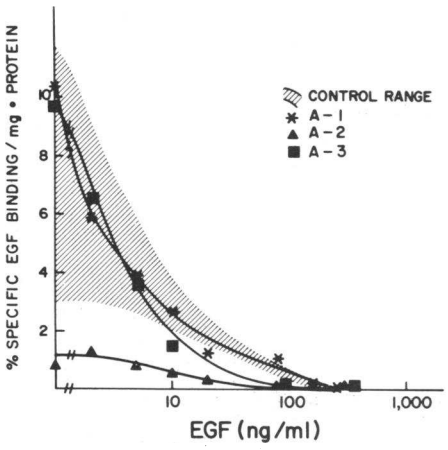

Figure 3. Specific ^{125}I-EGF binding to fibroblasts of patients with type A syndrome. Details are as for Fig. 1 except that cells of patients A-1, A-2, and A-3 (Boston) were used.

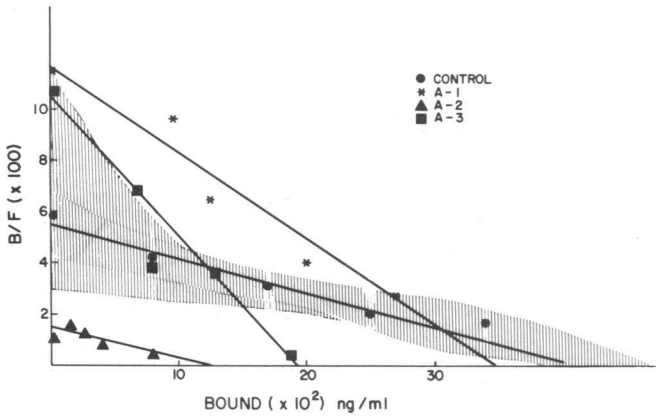

Figure 4. Scatchard analyses of EGF binding in cells of patients with type A syndrome. The data from Fig. 2 are replotted as bound/free (*B/F*) of EGF as a function of bound EGF.

three leprechaun cell lines. Stimulation was 60% of control in the cells of Ark-1, 40% in Can-1, and 18% in Minn-1. Considering the receptor numbers derived from Scatchard analysis, EGF receptor phosphorylation was reduced in parallel to reduction in receptor number in cells of Minn-1, but was reduced by a further 25% in cells of Ark-1 and Can-1. Basal levels of phosphorylation in both control and patients' cells were < 8% of stimulated values. The ^{32}P-labeled EGF receptor was of the same molecular mass in both patients and controls as determined by SDS-polyacrylamide gel electrophoresis.

In the type A patients' cell lines, EGF receptor phosphorylation ranged from 80% to 120% of control maximal levels (Fig. 7). Although cells of A-2 had estimated reduced EGF receptor number (25%), the levels of EGF receptor autophosphorylation were observed at essentially normal values. Patient A-3 is a patient whose cells demonstrated normal insulin binding and reduced insulin receptor phosphorylation previously. Again, there were no differences in basal levels of receptor phosphorylation between type A cells and controls.

Using anti-phosphotyrosine antibodies to immunoprecipitate the ^{32}P-labeled proteins, several other phosphoproteins were detected in EGF-stimulated cells (Fig. 6). These have sizes of 120, 98, 40, and 35 kD and are believed to represent endogenous substrates for the EGF receptor. The 120- and 35-kD phosphoproteins have been previously reported in other cell lines. ^{32}P incorporation into these proteins was significantly decreased in the cells of Ark-1 and Minn-1. In the cells of Can-1 there was increased basal levels of phosphorylation that reduced the amount of EGF stimulation observed. An increase in basal phosphorylation of the insulin receptor was also evident in wheat germ–purified preparation of cells of Can-1. It is not clear if this increase in basal phosphorylation was due to the EGF or insulin receptors, or to some other kinases. Substrate proteins, which are minimally tyrosine-phosphorylated, may be immunoprecipitated by the anti-phosphotyrosine antibody. In cells of Ark-1, phosphorylation of these presumed substrates was decreased even relative to receptor phosphorylation (85% vs. 40% decrease). In cells of Minn-1, stimulation of substrate phosphorylation paralleled the decreased receptor phosphorylation.

Sphingosine effects. The presence of decreased EGF binding and kinase activity, as well as our previous observation of decreased insulin receptor autophosphorylation and kinase ac-

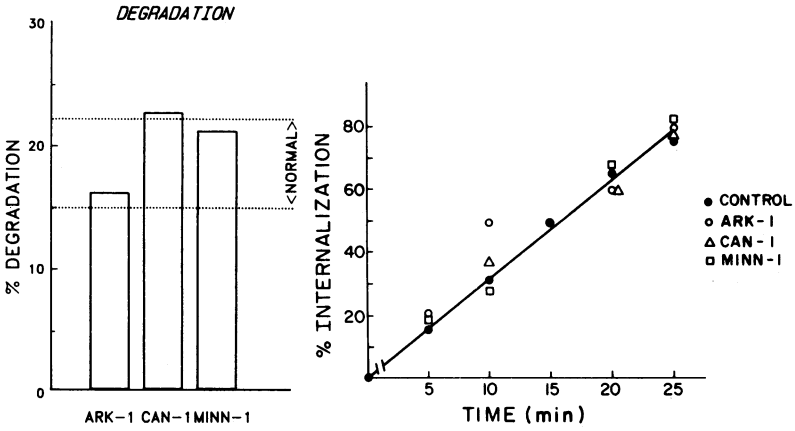

Figure 5. EGF internalization and degradation in fibroblasts of leprechaunism. Steady-state EGF binding was achieved at 4°C and the cells were then placed at 37°C. Up to 25 min, a linear time course of EGF internalization, as determined by the acid wash technique, was evident in both control (●) and patients' cell lines (*left panel*). (*Right panel*) The degree of degradation of internalized tracer at 20 min in the patients' cells is shown with the normal range indicated by the dashed lines.

tivity suggested the possible presence of an alteration in a common regulator of these receptors such as protein kinase C. Thus, the effects of sphingosine, a potent protein kinase C inhibitor, on EGF receptor function were examined in intact cells.

The fibroblasts were incubated with 40 μM sphingosine at 37°C for 30 min to allow interaction/incorporation into the cells before the binding and receptor phosphorylation assays. The binding studies were performed at 4°C to minimize internalization and were only of 3 h in duration (Fig. 8). In the control cells (n = 4), specific maximal EGF binding increased from 4% to 9.5%/mg protein. The levels of nonspecific binding were not affected. Scatchard analysis revealed that the increase in binding was due to an increase in affinity of the receptors with little change in receptor number. Similar effects were observed in the two leprechaun cell lines studied. In cells of Can-1 which normally express a decrease in receptor affinity rather than receptor number, sphingosine increased specific EGF binding from 2% to 18%/mg protein with a major increase in receptor affinity. In cells of Minn-1 which exhibit a defect in receptor number, sphingosine also increased EGF binding from 0.8% to 5%/mg protein owing to an increase in receptor affinity. Similar results were obtained after protein kinase C down regulation with 48 h of incubation with 1 μM phorbol 12,13-dibutyrate (data not shown). Sphingosine treatment did not have an effect on insulin binding.

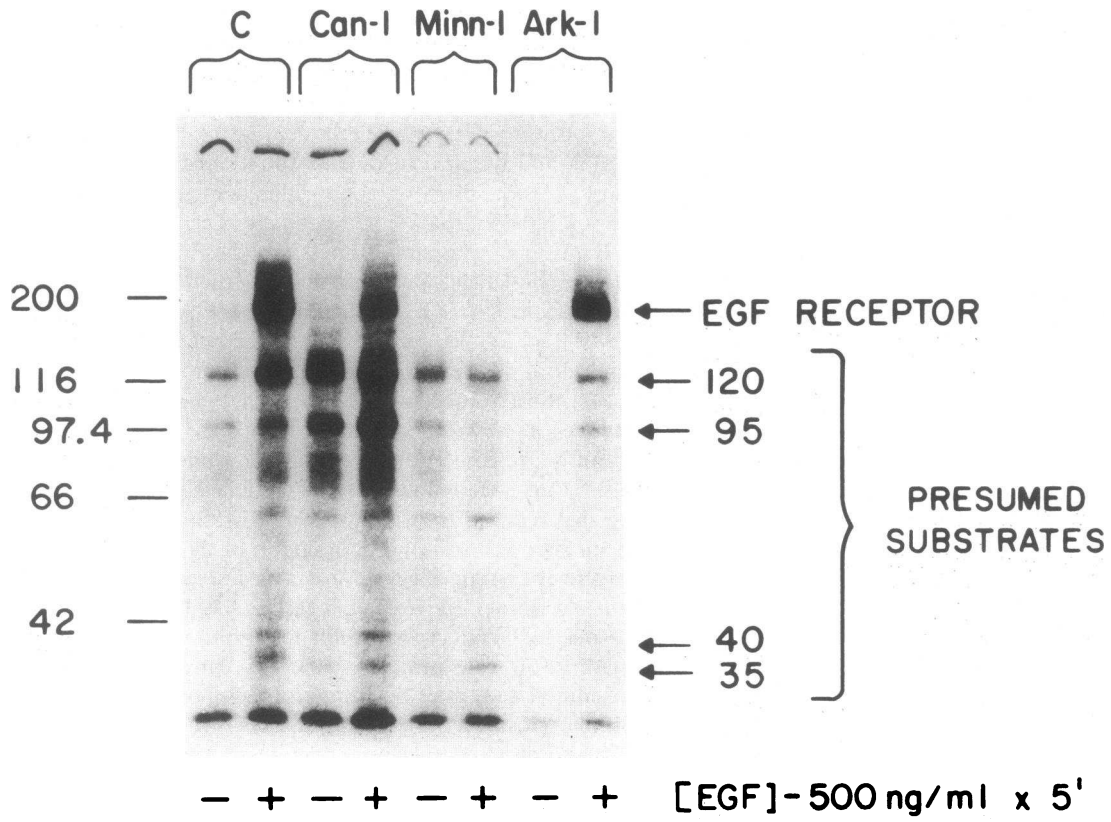

Figure 6. EGF stimulation of protein phosphorylation in intact fibroblasts of leprechaunism. ^{32}P-labeled cells were stimulated with 500 ng/ml of EGF for 5 min at 37°C; Triton X-100 cell extracts were prepared and incubated with polyclonal anti-phosphotyrosine antibody. The immunoprecipitates were separated by a 7.5% SDS–PAGE and analyzed by autoradiography. The phosphorylated EGF receptor and several presumed substrates (in kilodaltons) are denoted.

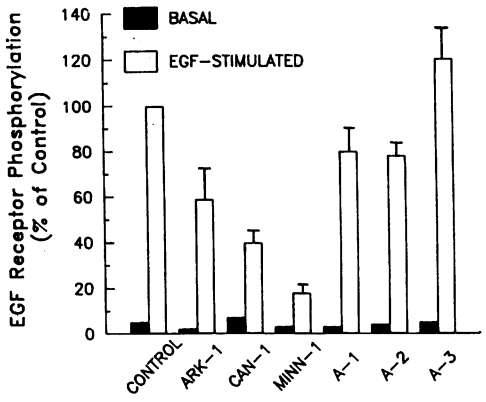

Figure 7. EGF receptor autophosphorylation in fibroblasts of leprechaunism and type A syndrome. Summary of scanning densitometry data of basal and stimulated EGF receptor phosphorylation in the patient cell lines. Data from three to five experiments per patient are graphed as a percentage±SEM of control maximum.

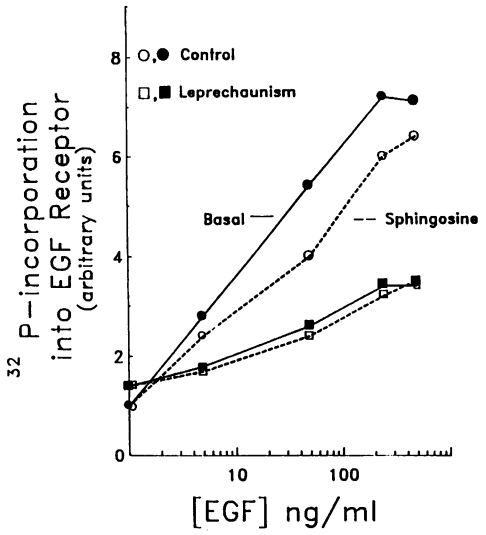

Figure 9. Effect of sphingosine on EGF receptor phosphorylation in fibroblasts of leprechaunism. Dose-response curves of EGF stimulated receptor phosphorylation (determined by scanning densitometry of autoradiograms) were generated in control cell lines (*circles*) and cells of leprechaunism (*squares*), with (*dashed lines*) and without (*solid lines*) sphingosine incubation.

The effect of sphingosine on EGF receptor autophosphorylation in control and patient cell lines is shown in Fig. 9. With the increased receptor affinity, one would expect to observe an increased sensitivity to EGF stimulation. However, at all doses of EGF, receptor autophosphorylation was not improved by sphingosine pretreatment. In fact, EGF receptor phosphorylation was slightly reduced when compared with control levels at the higher concentration of EGF. Similar results were obtained with the patient cell lines.

Discussion

Our objective was to examine EGF receptor function in cultured fibroblasts of patients with leprechaunism, Ark-1, Can-1, and Minn-1. These cell lines were derived from three unrelated patients born with typical features of leprechaunism. We and others have previously shown that these cells are abnormal with respect to insulin receptor function. All three patient cell lines demonstrated insulin binding < 15% of control. When normalized for insulin binding capacity, in vitro insulin receptor autophosphorylation was reduced in cells of Ark-1 and Minn-1, and the insulin receptor tyrosine kinase activity on exogenous substrates was found to be reduced in all three cell lines (8).

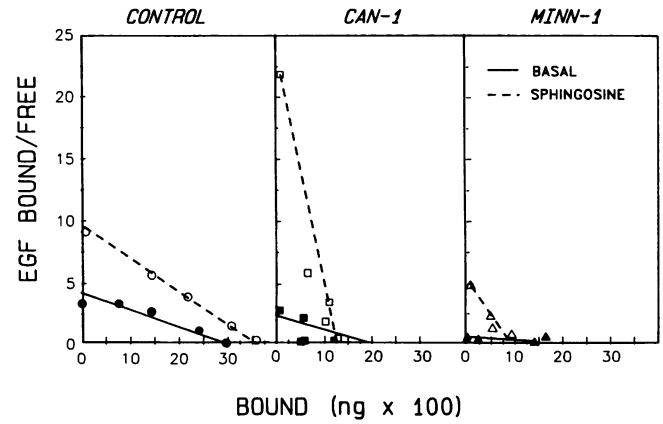

Figure 8. Effect of sphingosine on EGF binding in two fibroblast cell lines of leprechaunism (Can-1 and Minn-1). Scatchard analyses of EGF binding with (*dashed lines*) and without (*solid lines*) incubation with sphingosine are depicted for control cells, cells of Can-1, and cells of Minn-1 in the three panels.

Up to the present, there have been limited studies of other tyrosine kinase linked growth factors, such as insulin-like growth factor I (IGF-I), multiple stimulating activity (MSA), and EGF, in fibroblasts of patients with leprechaunism (5, 20–22). Recently, there has been a report of elevated levels of EGF in the urine of one patient with leprechaunism (Ark-1) (22). The cells of Ark-1 have been reported to have reduced binding and actions of MSA, whereas cells of Can-1 had reduced binding of IGF-1 and MSA but apparently normal EGF binding (20, 21). The latter data are discordant with the results of the present study and may be due to the fact that, in the previous study, the EGF binding was performed at 37°C where internalization can account for much of the cell-associated hormone. The cells of NC-1 (another patient with leprechaunism) had reduced biological effects of insulin, IGF-I and EGF but their binding parameters were normal (5). When studying skin fibroblasts, there is always a question of the overlapping specificity of insulin and IGF-I receptors, and their effects on data interpretation. Thus there is suggestion of a defect in signal transduction of several growth factors.

The present study shows that cells from three patients with leprechaunism all exhibit abnormal EGF receptor function. The cells of Minn-1 have a severe reduction of EGF receptors (25% of control) and a parallel decrease in EGF receptor autophosphorylation. The cells of Ark-1 and Can-1 exhibit a modest reduction in receptor number, as well as in affinity, accompanied by EGF receptor autophosphorylation reduced to more than expected levels.

Despite the reduced EGF receptor phosphorylation in the cells of leprechaunism, when corrected for amount of EGF binding, EGF internalization and degradation proceeded at normal rates. Similar findings were reported for insulin as well. Insulin internalization and degradation at 30 min at 37°C in these same cell lines were within the normal range. Livneh et

al. (23, 24) have recently studied Chinese hamster ovary cells transfected with cDNA constructs of EGF receptor mutants lacking tyrosine kinase activity; EGF receptor internalization rates and amount of degradation were also normal. Our results in these cell lines of leprechaunism are consistent with this finding and suggest that EGF receptor phosphorylation is not required for internalization.

There are several possible hypotheses regarding the multiple receptor abnormalities observed in leprechaunism. These include (*a*) the presence of isolated defects in several receptors, (*b*) the presence of an intracellular enzyme defect or a cell membrane defect affecting multiple receptors, or (*c*) insulin resistance directly or indirectly leading to regulation of other receptors' function.

Our results with wheat germ–purified preparations of insulin receptor from fibroblasts of leprechaunism (8), as well as recent sequence data on the insulin receptor cDNA from EB virus transfected lymphocytes of Ark-1 (25), suggest that the insulin receptor abnormality in at least some patients is due to a genetic defect in the insulin receptor. Based on cloning and sequencing data, Ark-1 is thought to have a paternal allele coding for a truncated receptor protein (not expressed at the cell surface) and a maternal allele coding for a single amino acid change in the α-subunit of the receptor which leads to subtle insulin binding abnormalities.

In search of possible mechanisms for the altered EGF receptor function, we have performed studies in which conditioned media from these cell lines are added to control cell lines, but have not found any secreted inhibitors of EGF binding (data not shown). It is possible that the insulin resistance present in leprechaunism may lead to altered plasma membrane composition which in turn may affect multiple receptors. Phosphatase activity may be playing a role also but studies of cells of Minn-1 in the presence of 100 μM sodium orthovanadate, a potent phosphotyrosine phosphatase inhibitor, revealed only minimal enhancement of EGF receptor phosphorylation.

Protein kinase C has been shown to alter EGF receptor binding affinity, as well as internalization and kinase activity of the receptor (12, 26). The insulin receptor kinase is also regulated by protein kinase C (27, 28). A spectrum of insulin actions are affected by phorbol esters and by down-regulation of protein kinase C (28, 29). A synthetic diacylglycerol, 1-oleol-2-acetyl glycerol, rapidly decreased the affinity of the EGF receptors in Swiss 3T3 cells and phorbol esters have been found to similarly affect EGF receptor function (26).

To test the possible role of protein kinase C in these multiple receptor abnormalities we have used sphingosine in some of the binding and phosphorylation experiments. Sphingosine is the predominant long-chain base in cell membranes and can be generated from the breakdown of sphingolipids (13). Sphingosine and some of its analogues have been found to be potent reversible inhibitors of protein kinase C by blocking phorbol ester binding and competes with diacylglycerol and Ca^{2+} for protein kinase C (30–35). Recently, it has been reported that, in WI-38 human fetal lung fibroblasts (35), sphingosine (5 μM) increased EGF receptor affinity and stimulated EGF receptor autophosphorylation and EGF receptor kinase activity in the absence of EGF. Different cell lines may respond differently to sphingosine. We therefore attempted to determine whether the EGF receptor dysfunction in the cells of leprechaunism is fixed or reversed by inhibiting protein kinase C activity. Sphingosine increased EGF binding in control cell lines as well as the patients' cell lines by increasing receptor affinity. However, the fold increase was far greater in the patients' cell lines. Although sphingosine improved EGF binding it did not augment EGF receptor autophosphorylation. In fact, in cells of both normals and patients with leprechaunism, EGF receptor phosphorylation was lower or similar to control levels. No shift to the left was evident in dose response curves of EGF stimulation of sphingosine-treated cells. Theoretically, protein kinase C inhibition should also enhance EGF receptor autophosphorylation; but there are some reports suggesting that sphingosine may not only inhibit protein kinase C but interact with the cell membrane in a less specific way (36, 37). Regardless of mechanism, it is clear that sphingosine did not correct the major abnormalities in EGF receptor function. Thus, although protein kinase C activity may be playing a role in the multiple defects observed, it is not likely to be the only site of pathology in leprechaunism.

An interesting question raised by these and previous data is whether insulin resistance can lead to modulation of other receptors' function. Recently, evidence of EGF receptor abnormalities in other models of diabetes has been reported. Ob/ob and db/db mice were found to have a 70–80% decrease in EGF binding in liver membranes, associated with decreased EGF receptor phosphorylation and decreased EGF receptor mRNA levels (38). Restriction fragment length analysis of the EGF receptor DNA revealed no abnormalities. Normal EGF binding was observed in cultured peritoneal fibroblasts from ob and db mice implying an acquired defect in these mice. Decreases in liver membrane EGF binding have also been noted in fa/fa Zucker rats, another animal model of hyperinsulinemic obesity. In our own laboratory, EGF binding and receptor phosphorylation was observed to be reduced in liver membranes from streptozotocin-treated diabetic rats (39). In pregnant, streptozotocin-treated diabetic rats, there was a 30% reduction in numbers of EGF receptors in placental membranes and a twofold elevation in circulating fetal EGF levels.

The type A syndrome of insulin resistance and acanthosis nigricans is known to have a genetic form of insulin resistance. Stimulation was 60% of control stimulation in the cells of Ark-1, 40% in Can-1, and 18% in Minn-1. We have had the opportunity to study three such patients (A-1, A-2, and A-3) in our laboratory. The erythrocytes of A-1 and A-2 had reduced insulin receptor number and affinity, respectively, whereas the erythrocytes and cultured fibroblasts of A-3 had normal insulin binding but reduced insulin receptor autophosphorylation. Our present data from the EGF receptor studies of the fibroblasts of these type A patients suggest that EGF receptor abnormalities are not necessarily due to the insulin resistance. Patient A-3's fibroblasts have been previously shown to have a insulin receptor kinase defect but have high normal EGF binding and normal EGF receptor phosphorylation. Interestingly, patient A-2's cells which had low EGF binding had almost normal levels of EGF-stimulated receptor phosphorylation. In view of these data, one must be cautious about a direct correlation between receptor number and receptor autophosphorylation. It is possible that there may exist a pool of EGF receptors which autophosphorylate upon EGF binding and a pool which does not, and that these two pools are differentially regulated. Cells of Minn-1 which had similar number of EGF receptors to those of A-2 had only 20% of control EGF receptor phosphorylation. The above findings suggest that type A

syndrome is heterogeneous in EGF binding parameters and that a defective insulin receptor kinase does not automatically imply EGF receptor dysfunction.

In conclusion, we have shown for the first time, abnormalities of two different growth factor receptors tyrosine kinases in cultured cells of patients with leprechaunism. Thus, regardless of the primary genetic lesion, leprechaunism appears to have more than just an isolated insulin receptor defect. A distinguishing feature between leprechaunism and type A syndrome may be the reduced EGF receptor autophosphorylation in the cells of leprechaunism. The results in this report lead us to propose that a more fundamental defect beyond the insulin receptor alone, is the probable cause of leprechaunism.

Acknowledgements

The authors wish to thank Dr. M. F. White for his generous support and Terri-Lyn Bellman for excellent secretarial assistance.

This work was supported by a Diabetes Canada Research Fellowship (SSKR) and by NIH grants DK 31036 and DERC grant DK 36836 from National Institutes of Health (CRK) and by the Pfizer Biomedical Research Award (CRK).

References

1. Donahue, W. L., and I. Uchida. 1954. Leprechaunism: euphemism for a rare familial disorder. *J. Pediatr.* 45:505–519.

2. Schilling, E. E., M. M. Rechler, C. Grunfeld, and A. M. Rosenberg. 1979. Primary defect of insulin receptor in skin fibroblasts cultured from an infant with leprechaunism and insulin resistance. *Proc. Natl. Acad. Sci. USA.* 76:5877–5881.

3. Knight, A. B., M. M. Rechler, J. A. Romanus, E. E. Van Oberghen-Schilling, and S. P. Nissley. 1981. Stimulation of glucose incorporation and amino acid transport by insulin and insulin like growth factor in fibroblasts with defective insulin receptors cultured from a patient with leprechaunism. *Proc. Natl. Acad. Sci. USA.* 78:2554–2558.

4. Podskalny, J. M., and C. R. Kahn. 1982. Cell culture studies on patients with extreme insulin resistance. II. Abnormal biological responses in cultured fibroblasts. *J. Clin. Endocrinol. Metab.* 54:269–275.

5. Kaplowitz, P. B., and A. J. D'Ercole. 1982. Fibroblasts from a patient with leprechaunism are resistant to insulin, epidermal growth factor and somatomedin C. *J. Clin. Endocrinol. Metab.* 55:741–748.

6. Taylor, S. I., B. Samuels, J. Roth, M. Kasuga, J. A. Hedo, P. Gorden, D. E. Brasel, T. Pokura, and R. R. Engel. 1982. Decreased insulin binding in cultured lymphocytes from two patients with extreme insulin resistance. *J. Clin. Endocrinol. Metab.* 54:919–930.

7. Grigorescu, F., M. F. White, and C. R. Kahn. 1983. Insulin binding and insulin dependent phosphorylation of the insulin receptor solubilized from human erythrocytes. *J. Biol. Chem.* 258:13708–13716.

8. Reddy, S. S.-K., V. Lauris, and C. R. Kahn. 1988. Insulin receptor function in fibroblasts from patients with leprechaunism: differential alterations in binding, autophosphorylation, kinase activity and receptor mediated internalization. *J. Clin. Invest.* 82:1359–1365.

9. Reddy, S. S.-K., D. Muller-Wieland, K. Kriauciunas and C. R. Kahn. 1989. Molecular defects in the insulin receptor in patients with leprechaunism and their parents. *J. Clin. Lab. Med.* In press.

10. Thompson, D. M., and G. N. Gill. 1985. The EGF receptor: structure, regulation and potential role in malignancy. *Cancer Surv.* 4:767–788.

11. Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tan, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid cell Ca cells. *Nature (Lond.).* 309:418–425.

12. Downward, J., M. D. Waterfield, and P. J. Parker. 1985. Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. *J. Biol. Chem.* 260:14538–14546.

13. Hannun, Y. A., and R. M. Bell. 1987. Lysosphingolipids inhibit protein kinase C: implications for the sphingolipidoses. *Science (Wash. DC).* 235:670–674.

14. Grigorescu, F., J. S. Flier, and C. R. Kahn. 1986. Characterization of binding and phosphorylation defects of erythrocyte insulin receptors in the type A syndrome of insulin resistance. *Diabetes.* 35:127–138.

15. Grigorescu, F., V. Herzberg, G. King, M. Meistas, J. Elders, T. Frazer, and C. R. Kahn. 1986. Defects in insulin binding and autophosphorylation of erythrocyte insulin receptors in patients with syndromes of severe insulin resistance and their parents. *J. Clin. Endocrinol. Metab.* 64:549–556.

16. Hammons, G. T., R. M. Smith, and L. Jarett. 1982. Inhibition by bacitracin of rat adipocyte plasma membrane degradation of ^{125}I-insulin is associated with an increase in plasma membrane bound insulin and a potentiation of glucose oxidation by adipocytes. *J. Biol. Chem.* 257:11563–11570.

17. Pang, D. T., B. R. Sharma, J. A. Shafer, M. F. White, and C. R. Kahn. 1985. Predominance of tyrosine phosphorylation of insulin receptors during the initial response of intact cells to insulin. *J. Biol. Chem.* 260:7131–7136.

18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–682.

19. Decker, S. J. 1984. Effects of epidermal growth factor and 12-*O*-tetra decanoylphoybol-13-acetate on metabolism of the epidermal growth factor receptor in normal human fibroblasts. *Mol. Cell. Biol.* 4:1718–1724.

20. Craig, J. W., J. Larner, E. F. Locker, B. Widom, and M. J. Elders. 1984. Mechanisms of insulin resistance in cultured fibroblasts from a patient with leprechaunism: impaired post-binding actions of insulin and multiplication-stimulating activity. *Metab. Clin. Exp.* 33:1084–1096.

21. Van Obberghen-Schilling, E. E., M. M. Rechler, J. A. Romanus, A. B. Knight, S. P. Nissley, and R. E. Humbel. 1981. Receptors for insulin-like growth factor I are defective in fibroblasts cultured from a patient with leprechaunism. *J. Clin. Invest.* 68:1356–1365.

22. Frindik, J. P., S. F. Kemp, R. H. Fiser, H. Schedewie, and M. J. Elders. 1985. Phenotypic expression in Donohue syndrome (leprechaunism): a role for epidermal growth factor. *J. Pediatr.* 107:428–430.

23. Livneh, E., R. Prywes, O. Kashles, N. Reiss, I. Sasson, Y. Mory, A. Ullrich, and J. Schlessinger. 1986. Reconstitution of human epidermal growth factor receptors and its deletion mutants in cultured hamster cells. *J. Biol. Chem.* 261:12490–12497.

24. Livneh, E., N. Reiss, E. Berent, A. Ullrich, and J. Schlessinger. 1987. An insertional mutant of epidermal growth factor receptor allows dissection of diverse receptor functions. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2669–2676.

25. Kadowaki, T., C. L. Bevins, A. Cama, K. Ojamaa, B. Marcus-Samuels, H. Kadowaki, L. Beitz, C. McKeon, and S. I. Taylor. 1988. Two mutant alleles of the insulin receptor gene in a patient with extreme insulin resistance. *Science (Wash. DC).* 240:787–790.

26. Sinnett-Smith, J. W., and E. Rozengurt. 1985. Diacylglycerol treatment rapidly decreases the affinity of the epidermal growth factor receptors of Swiss 3T3 cells. *J. Cell. Physiol.* 124:81–86.

27. Bollag, G. E., R. A. Roth, J. Beaudoin, D. Mochly-Rosen, and D. E. Koshland. 1986. Protein kinase C directly phosphorylates the insulin receptor in vitro and reduces its protein-tyrosine kinase activity. *Proc. Natl. Acad. Sci. USA.* 83:5822–5824.

28. Takayama S., M. F. White, V. Lauris, and C. R. Kahn. 1984. Phorbol esters modulate insulin receptor phosphorylation and insulin

action in cultured hepatoma cells. *Proc. Natl. Acad. Sci. USA.* 81:7797–7801.

29. Hachiya, H. L., S. Takayama, M. F. White, and G. L. King. 1987. Regulation of insulin receptor internalization in vascular endothelial cells by insulin and phorbol ester. *J. Biol. Chem.* 262:6417–6424.

30. Hannun, Y. A., C. R. Loomis, A. H. Merrill, and R. M. Bell. 1986. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J. Biol. Chem.* 261:12604–12609.

31. Merrill, A. H. Jr., A. M. Sereni, V. L. Stevens, Y. A. Hannun, R. M. Bell, and J. M. Kinkade, Jr. 1986. Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. *J. Biol. Chem.* 261:12610–12615.

32. Wilson, E., M. C. Olcott, R. M. Bell, A. H. Merrill, Jr., J. D. Lambeth. 1986. Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. *J. Biol. Chem.* 261:12616–12623.

33. Hannun, Y. A., C. S. Greenberg, and R. M. Bell. 1987. Sphingosine inhibition of agonist-dependent secretion and activation of human platelets implies that protein kinase C is a necessary and common event of the signal transduction pathways. *J. Biol. Chem.* 262:13620–13626.

34. Nelson, D. H., and D. K. Murray. 1986. Sphingolipids inhibit insulin and phorbol ester stimulated uptake of 2-deoxyglucose. *Biochem. Biophys. Res. Commun.* 138:463–467.

35. Davis, R. J., N. Girones, and M. Faucher. 1988. Two alternative mechanisms control the interconversion of functional states of the epidermal growth factor receptor. *J. Biol. Chem.* 263:5373–5379.

36. Pittet, D., K-H. Krause, C. B. Wollheim, R. Bruzzone, and D. P. Lew. 1987. Nonselective inhibition of neutrophil functions by sphinganine. *J. Biol. Chem.* 262:10072–10076.

37. Bazzi, M. D., and G. L. Nelsestuen. 1987. Mechanism of protein kinase C inhibition by sphingosine. *Biochem. Biophys. Res. Commun.* 146:203–207.

38. Blackshear, P. J., D. J. Stumpo, E. A. Kennington, J. S. Tuttle, D. N. Orth, K. L. Thompson, M-C. Hung, and M. R. Rosner. 1987. Decreased levels of hepatic epidermal growth factor receptors in obese hyperglycemic rodents. *J. Biol. Chem.* 262:12356–12364.

39. Sissom, J. F., W. K. Stenzel, and J. B. Warshaw. 1987. Decreased binding of epidermal growth factor in placentas from streptozotocin-diabetic rats. *J. Clin. Invest.* 80:242–247.