Involvement of the Ras/extracellular signal-regulated kinase signalling pathway in the regulation of ERCC-1 mRNA levels by insulin

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Expression of DNA repair enzymes, which includes ERCC-1, might be under the control of hormonal and growth factor stimulation. In the present study it was observed that insulin increased ERCC-1 mRNA levels both in Chinese hamster ovary cells overexpressing human insulin receptors (HIRc cells) and in fully differentiated 3T3-L1 adipocytes. To investigate the mechanisms underlying the increase in ERCC-1 gene expression in HIRc cells, we used a variety of pharmacological tools known to inhibit distinct signalling pathways. None of these inhibitors affected the amount of ERCC-1 mRNA in unstimulated cells. The pretreatment of cells with two chemically unrelated phosphatidylinositol 3'-kinase inhibitors, wortmannin and LY294002, failed to block the doubling of ERCC-1 mRNA content by

insulin. Similarly, inhibition of pp70 S6 kinase by rapamycin had no apparent effects on this insulin response. In contrast, altering the p21^{ras}-dependent pathway with either manumycin, an inhibitor of Ras farnesylation, or PD98059, an inhibitor of the mitogen-activated protein kinase/extracellular signal-regulated protein kinase (ERK) kinase, suppressed the induction of ERCC-1 mRNA by insulin ($P < 0.001$). Furthermore inhibition of RNA and protein synthesis negatively regulated the expression of this insulin-regulated gene ($P < 0.005$). These results suggest that insulin enhances ERCC-1 mRNA levels by the activation of the Ras-ERK-dependent pathway without the involvement of the phosphatidylinositol 3'-kinase/pp70 S6 kinase.

INTRODUCTION

The human excision repair cross-complementing gene (ERCC-1) is a mammalian DNA repair gene [1] whose gene product has been shown to have a crucial role in the early excision step of damaged DNA by virtue of its intrinsic structure-specific nuclease activity [2–4]. Nucleotide excision repair genes including ERCC-1 are viewed as determinants of cellular sensitivity to a variety of lesions to DNA [5–7]. Because of its extensive N-terminal homology with the yeast RAD10 protein, ERCC-1 might also participate in repair mechanisms involving homologous recombination [8]. Furthermore an increase in the content of the p53 protein has been noted in mice deficient in ERCC-1, presumably as a result of an accumulation of DNA lesions [9]. Although the importance of ERCC-1 on cellular protection against DNA damage is well documented, the regulation of ERCC-1 gene expression remains largely unknown.

Insulin exerts its biological effects by binding to a transmembrane heterotetrameric receptor with intrinsic tyrosine kinase activity [10]. After activation of the tyrosine kinase of the insulin receptor, tyrosine phosphorylation of a discrete number of cellular proteins and the subsequent propagation of a downstream cascade of phosphorylation events contribute to the metabolic and mitogenic responses of insulin [11]. Tyrosine phosphorylation of insulin receptor substrate 1 allows its interaction with numerous Src homology 2 (SH2) domain-containing proteins, including the 85 kDa subunit of phosphatidylinositol 3'-kinase (PI 3'-kinase) and the guanine-nucleotide exchange factor Grb2/SOS complex. Previous studies have indicated that the inhibition of PI 3'-kinase activity by wortmannin or LY294002 can attenuate the stimulation of glucose transport and mitogenesis by insulin [12,13]. This lipid kinase is linked

to the regulatory cascade that controls pp70 S6 kinase [13–15], whose activation by insulin can be selectively blocked by rapamycin without affecting PI 3'-kinase activity [14–16]. Activation of the p21^{ras} (Ras) pathway is the second arm of a signal transduction route that has been implicated in the induction of gene expression by insulin [17,18]. The association of the adaptor protein Grb2/SOS complex with either phosphorylated insulin receptor substrate 1 or Shc proteins through an SH2 domain interaction [19,20] represents the initial step leading to Ras activation. The formation of the active Ras-GTP complex is followed by the rapid activation of cytosolic 42 kDa mitogen-activated protein (MAP) kinase [extracellular signal-regulated protein kinase (ERK) 1 and 2] cascade by sequential phosphorylation and activation of the proto-oncogene Raf-1, and MAP kinase kinase (MEK). The expression of dominant inhibitory mutants of Ras leads to the inhibition of insulin-stimulated gene expression [17] but has no effect in insulin metabolic signalling [15,21]. Selective inhibition of farnesyl protein transferase by compounds such as manumycin prevents the attachment of Ras proteins to the plasma membrane and adversely affects their activation by growth factors (reviewed in [22]). Recently, Goalstone and Draznin [23] demonstrated that stimulation of Ras farnesylation is correlated with increased farnesyl transferase activity in response to insulin.

Our recent study has demonstrated an increase in ERCC-1 expression in Chinese hamster ovary (CHO) cells transfected with large numbers of insulin receptors (HIRc cells), resulting in an increased resistance to UV-induced damage [24]. However, the signalling pathway(s) utilized by insulin to regulate expression of ERCC-1 mRNA are yet to be determined. Here we show that this hormone exerts a stimulatory effect on ERCC-1 gene expression both in HIRc cells and in fully differentiated 3T3-L1

Abbreviations used: ERCC-1, human excision repair cross-complementing gene; CHO, Chinese hamster ovary; PI 3'-kinase, phosphatidylinositol 3'-kinase; MAP, mitogen-activated protein; MEK, MAP kinase kinase; HIRc cells, CHO cells overexpressing human insulin receptors; ERK, extracellular signal-regulated protein kinase; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; IGF-1, insulin-like growth factor 1; PEPCK, phosphoenolpyruvate carboxykinase.

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adipocytes, making HIRc cells a valid experimental model to investigate further this action of insulin. The effects of several pharmacological agents on insulin-stimulated increases in ERCC-1 mRNA were examined. Treatment of cells with manumycin or PD98059, a specific inhibitor of ERK activation [25,26], suppressed the insulin-induced increase in ERCC-1 mRNA by inhibition of the Ras-ERK pathway. In contrast, wortmannin, LY294002 and rapamycin had no effect, which indicates that this insulin response does not involve PI 3'-kinase/pp70 S6 kinase activities. Taken together, these results suggest a role for ERK in the regulation of ERCC-1 gene expression by insulin.

MATERIALS AND METHODS

Cell culture

HIRc cells were obtained by stable transfection of CHO cells with a plasmid containing a neomycin resistance gene driven by the SV40 promoter and a plasmid encoding the minus exon 11 variant of the human insulin receptor [27]. This cell line was kindly provided by Dr. Morris F. White (Joslin Diabetes Center, Boston, MA, U.S.A.). Cells were grown on tissue culture plates in Ham's F-12 medium containing 100 i.u./ml penicillin, 100 µg/ml streptomycin and 10 % (v/v) fetal bovine serum, and maintained in a humidified atmosphere of air/CO₂ (19:1) at 37 °C. Confluent monolayers of 3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD, U.S.A.) were induced to differentiate into adipocytes in Dulbecco's modified Eagle's medium (Paragon, Baltimore, MD, U.S.A.) supplemented with 10 % (v/v) fetal bovine serum (Hyclone, Logan, UT, U.S.A.), methylisobutylxanthine, dexamethasone and insulin as previously described [28]. Insulin was withdrawn from the medium 4 days after initiating differentiation. Cells were then maintained in Dulbecco's modified Eagle's medium and 10 % (v/v) fetal bovine serum for an additional 4 days, with a medium change at 2 days.

Cell treatment

Confluent cells were washed twice with PBS before a 4 h incubation in serum-free medium supplemented with 0.1 % insulin-free BSA (Sigma Chemicals, St. Louis, MO, U.S.A.). Cells were then exposed to various inhibitors at the indicated concentrations for periods ranging from 30 min to 1 h. Then the medium was supplemented with 0.1 % insulin-free BSA in the absence or presence of 50 nM insulin (Calbiochem, La Jolla, CA, U.S.A.). After 16 h, cells were harvested for total RNA extraction. All inhibitors were dissolved in DMSO as stock solutions, and diluted on the day of the experiment. The final concentration of DMSO was less than 0.01 % (v/v).

The PI 3'-kinase inhibitors wortmannin and LY294002 were purchased from Sigma and Calbiochem respectively. The pp70 S6 kinase inhibitor rapamycin, the Ras farnesylation inhibitor manumycin and the MEK inhibitor PD98059 were from Calbiochem. The RNA polymerase II inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and the protein synthesis inhibitor cycloheximide were from Calbiochem and Sigma respectively.

RNA isolation and Northern blot analysis

Total cellular RNA was extracted by homogenizing cells in Trizol reagent (Gibco-BRL, Gaithersburg, MD, U.S.A.). The reagent, a monophasic solution of phenol and guanidine isocyanate, is an improvement on the single-step RNA isolation method developed by Chomczynski and Sacchi [29]. Briefly, 5×10^6 cells were lysed in 1 ml of Trizol reagent and 0.2 ml (HIRc

cells) or 0.4 ml (3T3-L1 adipocytes) of chloroform. After a 5 min incubation at room temperature, the cell lysates were collected and centrifuged at 12000 g for 15 min at 4 °C. The upper aqueous phase from the adipocyte lysates was transferred to a new tube and further extracted with phenol/chloroform/3-methylbutanol-1-ol (25:24:1, by vol.). RNA was precipitated from the aqueous phase by mixing with 1 vol. of propan-2-ol. After centrifugation, the pelleted RNA was washed once with 75 % (v/v) ethanol before its resuspension in 10 µl of diethyl pyrocarbonate-treated RNase-free water. RNA from each sample was heated in the presence of 17 % (v/v) formaldehyde and 50 % (v/v) formamide to 65 °C for 15 min and then applied to a 1.2 % (w/v) agarose gel containing 17 % (v/v) formaldehyde. Ethidium bromide staining helped to assess the integrity of the RNA and to estimate loading efficiencies. After electrophoresis, RNA was transferred from the gel to nylon membrane (Nytran; Schleicher & Schuell, Keene, NH, U.S.A.) by capillary action and fixed by UV cross-linking (Stratalinker; Stratagene, La Jolla, CA, U.S.A.). The blots from HIRc cells were hybridized with a 431 bp hamster ERCC-1 cDNA probe [24], followed with a 1.8 kb human β-actin cDNA probe (Clontech, Palo Alto, CA, U.S.A.). Both probes were labelled with [α -³²P]dCTP (Amersham, Arlington Heights, IL, U.S.A.) by the random-priming procedure with a Megaprime labelling kit (Amersham). The blots from 3T3-L1 adipocytes were hybridized with a 222 bp hamster ERCC-1 RNA probe labelled with [α -³²P]UTP (Amersham) by the T3 polymerase transcription procedure with a MAXIscrip kit (Ambion Inc., Austin, TX, U.S.A.). The *HincII*-linearized recombinant plasmid (pBluescript KS+) containing the 431 bp hamster ERCC-1 cDNA fragment was used as the template for the synthesis of ³²P-labelled ERCC-1 RNA probe.

ERCC-1 hybridization was performed in 2.5 × Denhardt's solution/0.1 % SDS/5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/50 % (v/v) formamide/100 µg/ml denatured, sheared, salmon testes DNA overnight at 42 °C followed by two washes for 5 min each in 2 × SSC at room temperature, and two 30 min washes in 0.2 × SSC/0.1 % SDS at 42 °C. Blots were subjected to autoradiography at -70 °C with BioMax-MR films (Kodak, Rochester, NY, U.S.A.) and two intensifying screens. The amount of ERCC-1 mRNA was quantified by electronic autoradiography with a Packard Instant-Imager (Meriden, CT, U.S.A.). After quantification, the blots were washed twice with 0.1 % SDS at 80 °C to remove the radiolabelled probe, and then rehybridized with the β-actin probe under the same conditions as for ERCC-1. The ERCC-1 value in each lane was normalized by dividing by the β-actin value in the same lane.

PI 3'-kinase activity

PI 3'-kinase activity was measured as described previously [30], with some minor modifications. Serum-deprived cells grown in 35 mm dishes were preincubated in the absence or presence of wortmannin (50 nM) or LY294002 (10 µM) for 30 min before the addition of insulin (50 nM). After 16 h, cells were lysed; the lysates were immunoprecipitated with a polyclonal anti-p85 antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.). L-α-Phosphatidylinositol (Sigma) was used as substrate in the kinase assay.

MAP kinase activity

ERK1/2 activity was measured as described [30], with some minor modifications. In brief, cells grown in 35 mm dishes were switched to medium without serum and then preincubated in the

absence or presence of manumycin ($10 \mu\text{M}$) or PD98059 ($50 \mu\text{M}$) for 1 h before the addition of insulin (50 nM). After 5 min, cells were lysed in ice-cold lysis buffer containing 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Nonidet P40, 0.25% sodium deoxycholate, 1 mM NaF, 10 mM sodium pyrophosphate, 0.15 mM Pefabloc® SC (Boehringer Mannheim), 1 mM Na_3VO_4 , 20 $\mu\text{g}/\text{ml}$ aprotinin and 10 $\mu\text{g}/\text{ml}$ leupeptin. The lysates were centrifuged at 14000 g for 20 min at 4°C , and equal amounts of protein from each clarified lysate were immunoprecipitated with a polyclonal anti-(rat ERK1/2) antibody (ERK1-CT; Upstate Biotechnology) and protein G-Plus/protein A-agarose (Oncogene Science, Manhasset, NY, U.S.A.) for 16 h. After a series of washes, the immune pellets were incubated with 10 $\mu\text{g}/\text{ml}$ myelin basic protein (Upstate Biotechnology) and 20 μM [$\gamma\text{-}^{32}\text{P}$]ATP for 15 min at 22°C . The reaction was stopped by addition of Laemmli sample buffer [31], and the samples were analysed by one-dimensional (Novex, San Diego, CA, U.S.A.) SDS/PAGE (4–20% gel) under reducing conditions followed by autoradiography.

p70^{S6K} immune complex kinase assay

Confluent cells grown in 60 mm dishes were treated with various factors as described in the figure legends and lysed at 4°C for 15 min in 1 ml of lysis buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, 40 mM β -glycerophosphate, 50 mM NaF, 1% (v/v) Triton X-100, 1 mM Na_3VO_4 , 0.15 mM Pefabloc® SC, 10 $\mu\text{g}/\text{ml}$ aprotinin, 5 $\mu\text{g}/\text{ml}$ leupeptin, 10 nM okadaic acid, 10 nM tautomycin and 10 nM cypermethrin. Equal amounts of protein from clarified lysates were immunoprecipitated with a sheep polyclonal anti-(rat p70^{S6K}) antibody (Upstate Biotechnology) for 3 h, after which protein G-Plus/protein A-agarose was added for a further 1 h. The immune complexes were washed three times in lysis buffer and once with kinase assay buffer [20 mM Hepes (pH 7.4)/10 mM MgCl_2 /1 mM dithiothreitol/10 mM β -glycerophosphate/10 nM okadaic acid/10 nM tautomycin/10 nM cypermethrin]. The kinase reaction was performed by resuspending the pellet in 25 μl of kinase assay buffer containing 0.2 mM S6 peptide substrate (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), 20 μM protein kinase C substrate inhibitor peptide (Santa Cruz Biotechnology), 25 $\mu\text{g}/\text{ml}$ cAMP-dependent protein kinase inhibitor peptide (Upstate Biotechnology), 20 μM [$\gamma\text{-}^{32}\text{P}$]ATP (10 c.p.m./fmol) for 20 min at 22°C . The reaction was stopped by addition of 1 vol. of 0.75% (v/v) orthophosphoric acid. Aliquots of the supernatant were spotted on Whatman P81 phosphocellulose paper, followed by a series of four 5 min washes in 0.75% orthophosphoric acid and a rapid immersion in acetone. Autoradiography was performed on the dried filters.

Statistical analysis

Results are presented as means $\pm$ S.E.M. Comparison between groups were made by analysis of variance coupled to Fisher's protected least-squares difference post-hoc test with STATVIEW II[®] software for Apple Macintosh computers (Abacus Concepts, Berkeley, CA, U.S.A.).

RESULTS

Effect of insulin on ERCC-1 mRNA expression

We have recently observed that supraphysiological concentrations of insulin stimulates expression of ERCC-1 mRNA in

Figure 1 Effect of insulin on the expression of ERCC-1 mRNA in HIRc cells

Confluent CHO/HIRc cells were incubated in serum-free Ham's F-12 medium for 3–4 h before the addition of a range of concentrations of insulin (0–100 nM) for 16 h. Total RNA was extracted and Northern blot analysis was performed with cDNA probes for ERCC-1 and β -actin (A). The signals associated with these mRNA species were quantified by electronic autoradiography and their values corrected by dividing by the β -actin value in the same lane (B). (A) Autoradiogram of a representative experiment; (B) Means $\pm$ S.E.M. for three independent experiments, where the relative expression of ERCC-1 mRNA in the absence of insulin was arbitrarily set at 1.0. * $P < 0.05$, *** $P < 0.001$, compared with unstimulated cells.

HIRc cells maintained in serum-free medium [24]. The insulin receptors bind insulin with high affinity; however, high insulin concentrations can interact heterologously with the closely related insulin-like growth factor 1 (IGF-1) receptor [32]. Thus the possibility exists that insulin might have exerted its signalling potential by binding to the IGF-1 receptor. Therefore a concentration–response relationship of insulin should determine whether the insulin receptor mediates the stimulation of ERCC-1 mRNA in HIRc cells.

Figure 1 illustrates a Northern blot analysis performed on total RNA from HIRc cells that were incubated in serum-free medium in the presence of a range of concentrations of insulin for 16 h. There was an approximate doubling of ERCC-1 mRNA levels at 25 nM insulin. The concentration of insulin giving rise to half-maximal response (approx. 2.5 nM) corresponded well to the dissociation constant (K_d) of binding of insulin to its own receptor [33].

The possibility exists that overexpression of the insulin receptors in HIRc cells confers aberrant gene regulation. This seems unlikely because examination of ERCC-1 mRNA expression by Northern analysis in fully differentiated 3T3-L1 adipocytes revealed a comparable stimulation of ERCC-1 mRNA by insulin (1.6-fold and 1.8-fold increases with 10 and 100 nM insulin respectively). This finding supports the notion that the use of HIRc cells is a valid experimental model for investigating the effects of pharmacological agents on the expression of this insulin-regulated gene.

Figure 2 Effect of wortmannin and LY294002 on basal and insulin-induced ERCC-1 mRNA and on PI 3'-kinase activity in HIRc cells

Serum-free HIRc cells were treated with 50 nM wortmannin (**A**) or 10 μ M LY294002 (**B**) for 30 min before the addition of 50 nM insulin. After 16 h, total RNA was extracted and Northern blot analysis was used to quantify ERCC-1 and β -actin mRNA as described in the legend to Figure 1. (**A**) The stimulation of ERCC-1 mRNA by insulin was resistant to wortmannin treatment. The results presented are representative of two separate experiments. (**B**) Insulin stimulation of ERCC-1 mRNA is via LY294002-independent pathways. Bars represent means $\pm$ S.E.M. for three independent experiments, where the relative expression of ERCC-1 mRNA in the unstimulated control was arbitrarily set at 1.0. $P = 0.001$ compared with the value obtained in the unstimulated control (a) or LY294002 alone (b). (**C**) Wortmannin and LY294002 inhibit insulin-stimulated PI 3'-kinase activity in HIRc cells. Cells maintained in serum-free medium were treated with wortmannin or LY294002 at the indicated concentrations for 30 min before the addition of insulin (50 nM) for 16 h. The cells were lysed; clarified lysates were immunoprecipitated with anti-p85 antibody. The immune pellets were analysed for PI 3'-kinase activity as described in the Materials and methods section. The results presented are representative of several experiments.

Effect of PI 3'-kinase inhibitors on basal and insulin-induced ERCC-1 mRNA

Activation of PI 3'-kinase by insulin has a major role in gene regulation [34,35]. Inhibition of PI 3'-kinase can be achieved by two chemically unrelated compounds, wortmannin and LY294002 [14]. Thus these inhibitors were used to determine the importance of PI 3'-kinase activation in insulin-mediated increase in ERCC-1 mRNA (Figure 2). Insulin treatment of HIRc cells for 16 h approximately doubled ERCC-1 mRNA levels. This effect of insulin was not blocked by 50 nM wortmannin (Figure 2A) nor by LY294002 at 10 μ M (Figure 2B). The basal amount of ERCC-1 mRNA was not affected by either PI 3'-kinase inhibitor. Under these experimental conditions, both wortmannin and LY294002 inhibited insulin-stimulated PI 3'-kinase activity (Figure 2C). Thus these findings suggest that PI 3'-kinase is not involved in the regulation of ERCC-1 mRNA by insulin.

Figure 3 Effect of rapamycin on basal and insulin-induced ERCC-1 mRNA and on pp70 S6K activity in HIRc cells

(**A**) Serum-free HIRc cells were treated with 20 nM rapamycin for 30 min before the addition of 50 nM insulin. After 16 h, total RNA was extracted and Northern blot analysis was used to quantify ERCC-1 and β -actin mRNA as described in the legend to Figure 1. Bars represent means $\pm$ S.E.M. for four independent experiments, where the relative expression of ERCC-1 mRNA in the unstimulated control was arbitrarily set at 1.0. $P < 0.005$ compared with the value obtained in the unstimulated control (a) or rapamycin alone (b). (**B**) Rapamycin blocks insulin-stimulated pp70 S6K activity in HIRc cells. Cells maintained in serum-free medium were treated with 20 nM rapamycin for 30 min before the addition of insulin (50 nM) for 10 min. The cells were lysed; clarified lysates were immunoprecipitated with anti-(pp70 S6K) antibody. The immune pellets were analysed as described in the Materials and methods section. The results presented are representative of two independent experiments.

Figure 4 Effect of manumycin on basal and insulin-induced ERCC-1 mRNA and on ERK activity in HIRc cells

(**A**) Serum-free HIRc cells were treated with 10 μ M manumycin for 60 min before the addition of 50 nM insulin. After 16 h, total RNA was extracted and Northern blot analysis was used to quantify ERCC-1 and β -actin mRNA as described in the legend to Figure 1. Bars represent means $\pm$ S.E.M. for three independent experiments, where the relative expression of ERCC-1 mRNA in the unstimulated control was arbitrarily set at 1.0. $P < 0.001$ compared with the value obtained in the unstimulated control (a) or with the insulin alone (c). (**B**) Manumycin blocks insulin-stimulated ERK activity in HIRc cells. Cells maintained in serum-free medium were treated with 10 μ M manumycin for 1 h before the addition of insulin (50 nM) for 5 min. The cells were lysed; clarified lysates were immunoprecipitated with anti-ERK1 antibody. The immune pellets were analysed as described in the Materials and methods section. The results presented are representative of four experiments.

Figure 5 Effect of PD98059 on basal and insulin-induced ERCC-1 mRNA and on ERK activity in HIRc cells

(A) Serum-free HIRc cells were treated with 50 μ M PD98059 for 60 min before the addition of 50 nM insulin. After 16 h, total RNA was extracted and Northern blot analysis was used to quantitate ERCC-1 and β -actin mRNA as described in the legend to Figure 1. Bars represent means $\pm$ S.E.M. for three independent experiments, where the relative expression of ERCC-1 mRNA in the unstimulated control was arbitrarily set at 1.0. $P < 0.001$ compared with the value obtained in the unstimulated control (a) or with the insulin alone (c). (B) PD98059 blocks insulin-stimulated ERK activity in HIRc cells. Cells maintained in serum-free medium were treated with 50 μ M PD98059 for 1 h before the addition of insulin (50 nM) for 5 min. The cells were lysed; clarified lysates were immunoprecipitated with anti-ERK1 antibody. The immune pellets were analysed for ERK activity as described in the Materials and methods section. The results presented are representative of several experiments.

Effect of rapamycin on basal and insulin-induced ERCC-1 mRNA

Rapamycin prevents the activation of pp70 S6K by insulin without affecting PI 3'-kinase activity or the activity of other insulin-stimulated protein kinases [36,37]. Therefore we used this inhibitor to determine whether a rapamycin-sensitive pathway is involved in ERCC-1 gene induction by insulin (Figure 3A). ERCC-1 mRNA was not affected by rapamycin, either in unstimulated cells or in cells incubated with insulin. Under these experimental conditions, rapamycin inhibited insulin-stimulated pp70 S6K activity (Figure 3B). Therefore, as with the inhibitors of PI 3'-kinase, rapamycin did not prevent the insulin-induced increase in ERCC-1 mRNA.

Effect of manumycin and PD98059 on basal and insulin-induced ERCC-1 mRNA

Attachment of Ras protein to the plasma membrane by the enzyme farnesyl protein transferase is required for effective Ras signalling. The antibiotic manumycin is a farnesylation inhibitor that has been shown to suppress Ras activity selectively in whole cells [38,39]. Therefore we used manumycin to test whether insulin-induced ERCC-1 gene expression is a Ras-mediated event (Figure 4A). Manumycin at 10 μ M had no effect on basal ERCC-1 mRNA, but totally blocked the stimulation of ERCC-1 mRNA by insulin ($P < 0.001$).

The inhibition of insulin-stimulated Ras activity in cells exposed to a farnesylation inhibitor [40] results in alterations of

Figure 6 Manumycin and PD98059 block ERCC-1 mRNA expression induced by insulin in differentiated 3T3-L1 adipocytes

Serum-free 3T3-L1 adipocytes were treated with vehicle (lanes 1 and 2), 10 μ M manumycin (lanes 3 and 4) or 50 μ M PD98059 (lanes 5 and 6) for 60 min before the addition of 50 nM insulin where indicated. After 16 h, total RNA was extracted and Northern blot analysis was performed with a 32 P-labelled 222 bp ERCC-1 RNA probe as described in the Materials and methods section. The results presented are representative of two experiments.

Table 1 Effect of transcription and translation inhibitors on basal and insulin-induced ERCC-1 mRNA levels in HIRc cells

Serum-free HIRc cells were treated with 100 μ M DRB or 5 μ M cycloheximide for 30 min before the addition of 100 nM insulin. After 16 h, total RNA was extracted and Northern blot analysis was used to quantify ERCC-1 mRNA. The results are expressed as percentages of the ERCC-1 mRNA values obtained in the unstimulated control and as means $\pm$ S.E.M. for three independent experiments.

Treatment	ERCC-1 mRNA levels (% of control)
Control	100
Insulin	152 $\pm$ 4
DRB	61 $\pm$ 13
DRB + insulin	76 $\pm$ 8
Cycloheximide	85 $\pm$ 10
Cycloheximide + insulin	102 $\pm$ 16

Ras signalling cascade, which includes the Raf-1/MEK/ERK pathway [38,40]. Indeed, treatment of HIRc cells with manumycin greatly decreased insulin-stimulated ERK1/2 activity (Figure 4B). To determine the importance of ERK activation in insulin-stimulated ERCC-1 mRNA expression we assessed the effect of PD98059. This agent is a selective inhibitor of MEK that phosphorylates and activates ERK. The pretreatment of cells with PD98059 at 50 μ M prevented the induction of ERCC-1 mRNA by insulin ($P < 0.001$), whereas the basal amount of ERCC-1 mRNA was not affected (Figure 5A). As expected PD98059 abrogated the insulin-stimulated ERK1/2 activity (Figure 5B). A similar picture was observed in 3T3-L1 adipocytes (Figure 6). Treatment with either manumycin or PD98059 drastically attenuated insulin's ability to increase ERCC-1 mRNA expression. Thus these findings suggest that the Ras-ERK pathway does have a major role in insulin-induced accumulation of ERCC-1 mRNA.

Effect of DRB and cycloheximide on basal and insulin-induced ERCC-1 mRNA

To gain additional insights into the mechanisms underlying the stimulatory effect of insulin on ERCC-1 mRNA level, cells were exposed to DRB, a potent inhibitor of RNA polymerase II [41], before a 16 h incubation in the absence or presence of insulin (Table 1). DRB at 100 μ M showed a significant (40%) inhibition on ERCC-1 mRNA in the absence of insulin ($P < 0.05$) and a

complete suppression of the action of insulin. Therefore early regulatory controls at transcriptional and post-transcriptional levels such as mRNA stability might participate in both basal and insulin-induced increase of ERCC-1 mRNA.

This action of insulin could depend on continuing protein synthesis. To verify this hypothesis, an inhibitor of protein synthesis, cycloheximide, was used. Cells maintained in serum-free medium were exposed to cycloheximide at 5 μ M for 30 min before the incubation in the absence or presence of insulin (Table 1). Cycloheximide completely inhibited the stimulatory effect of insulin ($P < 0.005$) without affecting basal ERCC-1 mRNA levels. These data suggest that synthesis of proteins *de novo* is required for mediation of the induction of ERCC-1 mRNA by insulin.

DISCUSSION

It has recently been demonstrated that transfection of the insulin receptors enhances the expression of ERCC-1 mRNA in CHO cells while conferring increased resistance to UV-induced damage [24]. Our new findings indicate that ERCC-1 is an insulin-regulated gene both in HIRc cells and 3T3-L1 adipocytes, a cell model known for its high responsiveness to insulin. The ability to introduce wild-type and mutated forms of the insulin receptors in a variety of cell types, including CHO cells, has helped to improve our understanding of insulin signal transduction. Indeed, it was shown that cells expressing a kinase-defective insulin receptor mutant were unable to transmit insulin signals to various biological endpoints (reviewed in [11]), which included the mRNA expression pattern for the ERCC-1 gene [24].

In the present study the signal transduction pathway used by insulin to induce accumulation of ERCC-1 mRNA was investigated. Various inhibitors of key steps thought to be required in insulin signalling have been used to address this question. This approach has previously provided important insight into the understanding of the role of signalling molecules in the regulation of several insulin-regulated genes. For instance, the PI 3'-kinase pathway has been clearly implicated by this approach as having an active role in the insulin regulation of the primary response gene cMG1, GLUT 1 and hexokinase II mRNA expression [42–44], and in the inhibition of glucocorticoid- and cAMP-stimulated phosphoenolpyruvate carboxykinase (PEPCK) gene expression by insulin [34]. Moreover, selective inhibition of the Ras–ERK pathway eliminates the ability of insulin to stimulate AP-1-dependent gene transcription [45] and GLUT 3 mRNA concentration [42] but fails to alter insulin antagonism of cAMP- and glucocorticoid-stimulated PEPCK gene transcription [46]. Direct demonstration of a role of a Ras-dependent signalling pathway for insulin-induced gene expression has been provided with the expression of dominant-negative mutants of Ras or MEK [17,45].

The results in the present study clearly demonstrate an important role for the Ras–ERK pathway in the accumulation of ERCC-1 mRNA by insulin both in HIRc cells and 3T3-L1 adipocytes. The pretreatment of cells with manumycin, a Ras farnesylation inhibitor, prevents this insulin action. Furthermore by blocking insulin-stimulated ERK activity, PD98059, a specific MEK inhibitor, attenuated insulin's ability to regulate ERCC-1 mRNA expression. This pathway has been previously implicated in the regulation of *c-fos* gene transcription by insulin [17,18], but not that of PEPCK or hexokinase II [34,44]. Hence the results presented here are consistent with the notion that the insulin-induced increase in ERCC-1 mRNA is a Ras/ERK-dependent event.

We did not observe a similar role for the PI 3'-kinase pathway.

Inhibition of insulin-stimulated PI 3'-kinase activity with either wortmannin or LY294002 failed to abolish the increase in ERCC-1 mRNA by insulin, suggesting that in HIRc cells there is no requirement for this lipid kinase in this action of insulin. It has been shown that after its activation, PI 3'-kinase can mediate the activation of pp70 S6 kinase [47]. By using rapamycin, an inhibitor that binds to the FRAP kinase and blocks pp70 S6 kinase activation [48], we analysed the role of this protein in insulin-mediated increase in ERCC-1 mRNA. We observed that rapamycin did not affect the insulin regulation of ERCC-1 mRNA expression. A similar lack of effect of rapamycin treatment has been reported for the regulation of PEPCK and GLUT 3 gene expression by insulin [34,43]. However, these results contrast with the findings of Osawa et al. [44] and Taha et al. [43], who showed that rapamycin can inhibit the insulin-mediated increase in hexokinase II and GLUT 1 mRNA. Indeed, a differential pattern of insulin regulation of specific genes can occur in the same cell [35,43,49]. Taken together, our results rule out a role for PI 3'-kinase and pp70 S6 kinase in the mechanism by which ERCC-1 mRNA is induced by insulin.

It has been shown that insulin affects glycogen synthase mRNA stability rather than transcription in cultured rat hepatoma cells [50], whereas transcriptional events have a determinant role in insulin-regulated expression of several genes, including the genes for *c-fos*, PEPCK, tyrosine aminotransferase and hexokinase II [18,44,49,51]. In the present study, the insulin-induced increase in ERCC-1 mRNA can be completely prevented by DRB, a potent inhibitor of mRNA synthesis. Work is under way in our laboratory to elucidate whether the accumulation of ERCC-1 mRNA observed with insulin is derived from an increase in RNA stability rather than from transcriptional activation.

Using a well-established protein synthesis inhibitor, we show that protein synthesis *de novo* has a major role in the insulin-mediated increase in ERCC-1 mRNA. The pretreatment of HIRc cells with cycloheximide abolishes the ability of insulin to stimulate ERCC-1 gene expression. These results suggest that this action of insulin requires short-lived protein(s). In contrast, it has been suggested that inhibition of translation can regulate transcription in an insulin-like manner [52]. Surprisingly, Bortoff and Messina observed a decrease in PEPCK transcription in cultured rat hepatoma cells after treatment with either cycloheximide or insulin. This disparity between the results of Bortoff and Messina and those reported here could be due to gene-specific regulation.

We used an anti-ERCC-1 antibody to detect the ERCC-1 protein by immunoblotting. Unfortunately, the amount of ERCC-1 protein expressed was below the detection limit of our assay, making it impossible to determine whether ERCC-1 protein levels were regulated in response to insulin. These results are in agreement with the finding of van Vuuren et al. [53], who showed instability of ERCC-1 protein due to rapid degradation *in vivo*. In any event, an excellent concordance between ERCC-1 mRNA expression and resistance to DNA damage has been demonstrated [54,55].

Normal cellular metabolism can generate oxidative damage that leads to DNA lesions [56]. The accumulation of unrepaired DNA damage leads to a general decrease in DNA synthesis, resulting in defects in nuclear and cell division. As a defence system, cells possess active DNA repair pathways such as the nucleotide excision repair. It is a complex process whereby lesions are located, a stretch of nucleotides containing the damage is excised and a repair patch is synthesized [57]. ERCC-1 is required for the incision step necessary to remove damaged DNA. Moreover, the homology of ERCC-1 with RAD10 is indicative of a role in homologous recombination. The notion

that hormones and growth factors could control expression of DNA repair genes in mammalian cells is of great interest. High glucose concentrations found in diabetic conditions result in DNA damage in a variety of cell types [58] associated with the perturbation of DNA repair synthesis [59]. Such impairment in DNA repair might result in aberrant gene expression, leading to degenerative disorders including diabetic microangiopathy. Efforts are under way in our laboratory to assess whether expression of DNA repair genes is altered in diabetic conditions.

In summary, this study addressed for the first time the mechanism by which ERCC-1 mRNA increases in response to insulin and found that this action of insulin is governed by a Ras-ERK-dependent event.

We thank Lisa G. Adams and Sutapa Kole for their expert technical assistance.

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