Homocysteine thiolactone inhibits insulin signaling, and glutathione has a protective effect

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ABSTRACT

Hyperhomocysteinemia and insulin resistance are independent factors for cardiovascular disease. Most of the angiotoxic effects of homocysteine are related to the formation of homocysteine thiolactone and the consequent increase in oxidative stress. The oxidative stress has also been shown to impair insulin action, therefore leading to insulin resistance. In order to study a putative direct effect of homocysteine on insulin signaling, we have characterized the molecular counter-regulation of the early events in the signal transduction of the insulin receptor, and the metabolic end-point of glycogen synthesis. We employed HTC rat hepatoma cells transfected with the human insulin receptor. A 10 min exposure to homocysteine thiolactone (50 μ M) resulted in a significant inhibition of insulin-stimulated tyrosine phosphorylation of the insulin receptor β -subunit and its substrates IRS-1 and p60-70, as well as their association with the p85 regulatory subunit of phosphatidylinositol 3-kinase.

These effects led to impairment of the insulin-stimulated phosphatidylinositol 3-kinase activity, which plays a central role in regulating insulin action. Thus, insulin-stimulated glycogen synthesis was also inhibited by homocysteine thiolactone. To investigate whether oxidative stress was mediating the counter-regulatory effect of homocysteine thiolactone on insulin signaling, we preincubated the cells (5 min) with 250 μ M glutathione prior to the incubation with homocysteine (10 min) and subsequent insulin challenge. Glutathione completely abolished the effects of homocysteine thiolactone on insulin-receptor signaling and restored the insulin-stimulated glycogen synthesis. In conclusion, these data suggest that homocysteine thiolactone impairs insulin signaling by a mechanism involving oxidative stress, leading to a defect in insulin action.

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INTRODUCTION

Elevated plasma levels of homocysteine are an independent risk factor for atherothrombosis (Boushey *et al.* 1995, Kang & Wong 1996, McCully 1996). Hereditary enzymatic deficiencies and nutritional deficiencies of folate, pyridoxine or cobalamin, as well as chronic renal failure, are associated with elevated blood homocysteine and accelerated atherosclerosis (Guttormsen *et al.* 1996, Jacques *et al.* 1996, Kang & Wong 1996, Robinson *et al.* 1996). The etiological factors for atherosclerosis are believed to increase conversion of methionine to homocysteine thiolactone, the reactive cyclic internal lactone of homocysteine (McCully 1993,

Jakubowski *et al.* 2000). The synthesis of homocysteine thiolactone occurs in all human cell types, and increased homocysteine levels lead to elevation of thiolactone levels in human cells (Jakubowski 2000). The oxidant stress of hyperhomocystein(e)mia, partly mediated by the production of thiolactone, seems to underly the vascular dysfunction produced by homocysteine (Blundel *et al.* 1996, Loscalzo 1996).

Another independent risk factor for atherosclerosis is insulin resistance (Ferrannini *et al.* 1991, Reaven 1993), and oxidative stress is thought to be a causal link (Ceriello & Pirisi 1995, Paolisso & Giuliano 1996, Wittmann & Nagy 1996). Thus, oxidative stress has been shown to reduce insulin

action *in vivo* (Nourooz-Zadeh *et al.* 1997, De Mattia *et al.* 1998) and *in vitro* (Rudich *et al.* 1997, Blair *et al.* 1999, Khamaisi *et al.* 2000), as well as insulin signaling (Blair *et al.* 1999, Hansen *et al.* 1999, Tirosh *et al.* 1999). Since oxidative stress is supposed to mediate the homocysteine effects on atherosclerosis, the question now arises as to whether homocysteine might affect insulin signaling, thereby providing a molecular mechanism for the induction of insulin resistance. Therefore, we proposed the hypothesis that, *in vitro*, homocysteine thiolactone could inhibit insulin signaling by inducing oxidative stress.

Insulin is known to activate multiple signaling pathways (Cheatham & Kahn 1995, Myers & White 1996), but the earlier events are the activation of tyrosine kinase activity of the insulin receptor β -subunit (White *et al.* 1988), the tyrosine phosphorylation of its substrates (Sun *et al.* 1992, Sung *et al.* 1994, Sánchez-Margalet & Najib 1999), and the interaction with SH2-domain-containing proteins, such as p85, the regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Sun *et al.* 1992, Sánchez-Margalet *et al.* 1995, Sánchez-Margalet & Najib 1999). PI3K plays a central role in regulating glucose transport and glycogen synthesis (Sánchez-Margalet *et al.* 1994, Sánchez-Margalet 2000). In this study, we show that short-term incubation with homocysteine thiolactone inhibits insulin-receptor signaling and insulin-stimulated glycogen synthesis. These effects can be prevented by the presence of glutathione, suggesting that oxidative stress might mediate the inhibition of insulin signaling and action.

MATERIALS AND METHODS

Antibodies and reagents

Monoclonal antibodies (anti-insulin receptor (α -IR) and anti-Sam68 (α -Sam68)) and polyclonal anti-IRS-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antiserum to the p85 α regulatory subunit of PI3K (α -p85) was from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Monoclonal antibodies to phosphotyrosine (α -PY) were purchased from Transduction Laboratories (Lexington, KY, USA). Electrophoretic chemicals and molecular weight standards were from Novex (San Diego, CA, USA). Fetal calf serum, culture medium (Dulbecco's modified Eagle's medium (DMEM)) and supplements (antibiotics, glutamine) were from Biological Industries (Kibbutz Beit Haemek, Israel). Protein-A Sepharose was from Amersham Pharmacia Biotech (Barcelona, Spain). Homocysteine thiolactone

was from Sigma (Alcobendas, Madrid, Spain). [U - ^{14}C]Glucose (250 mCi/mmol) was purchased from ICN Iberica (Barcelona, Spain).

Cells and preparation of soluble cell lysates

Rat HTC hepatoma cells overexpressing the human insulin receptor (HTC-IR) were kindly provided by Dr Ira D Goldfine (University of California, San Francisco, CA, USA). Cells were prepared and maintained in DMEM as previously described (Sánchez-Margalet *et al.* 1995). For experiments, cells were grown in 100 mm dishes to 90% confluency and serum-starved for 24 h. They were treated for 5 min at 37 °C with 100 nM insulin, and preincubated (or not) with 50 μ M homocysteine thiolactone for 10 min prior to insulin stimulation. In some experiments, 250 μ M glutathione was added 5 min before homocysteine thiolactone preincubation. Homocysteine and glutathione were not washed before insulin stimulation, and were present during the incubation period. Cells were solubilized for 30 min at 4 °C in lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM sodium orthovanadate (Sánchez-Margalet *et al.* 1995). After centrifugation, the soluble cell lysates were used for the study. The protein concentration was determined using a kit from Bio-Rad (Richmond, CA, USA), with bovine serum albumin as the standard.

Immunoprecipitation and Western blotting

Soluble cell lysates (2 mg protein) were first precleared with 50 μ l protein A-Sepharose for 2 h at 4 °C and incubated with appropriate antibodies for 2 h at 4 °C. Fifty microlitres protein A-Sepharose were then added to the antibody-containing sample and incubation was continued for 1 h at 4 °C (Sánchez-Margalet *et al.* 1995). The immunoprecipitates were washed three times with lysis buffer. Fifty microlitres SDS-stop buffer containing 100 mM DTT were added to immunoprecipitates and then boiled for 5 min. These were then analysed by Western blotting. Samples were resolved by SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The membranes were blocked with Tris-buffered saline/0.05% Tween 20 (TBST) containing 5% non-fat dried milk for 1 h, washed in TBST, and incubated with primary antibodies. After being washed in TBST, membranes were further incubated with secondary antibodies linked to horseradish peroxidase. Bound horseradish peroxidase was visualized by a highly

sensitive chemiluminescence system (SuperSignal; Pierce, Rockford, IL, USA) (Sánchez-Margalet & Najib 1999). The bands obtained in the Western blots were scanned and then analysed by the PCBAS2.0 program.

PI3K activity

PI3K activity was measured directly in anti-phosphotyrosine immunoprecipitates in 50 μ l of a reaction mix containing 0.2 mg phosphatidylinositol/ml (Sigma), 20 mM Hepes, pH 7.1, 0.4 mM EGTA, 0.4 mM sodium phosphate, 10 mM MgCl_2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (40 μ M and 0.1 μ Ci/ μ l) (Sung *et al.* 1994, González-Yanes & Sánchez-Margalet 2000). After 5 min, the reaction was stopped by the addition of 15 μ l 4 M HCl and 130 μ l chloroform/methanol (1:1). Twenty microliters of the lower organic layer was spotted onto a silica gel-60 plate (Merck, Darmstadt, Germany), which was preactivated with 1% potassium oxalate at 100 $^{\circ}\text{C}$ and analyzed by thin-layer chromatography in chloroform/methanol/water/ammonia (60:27:11:2 by vol.). Dried plates were then exposed to a film for 3–7 days with intensifying screens for autoradiography.

Glycogen synthesis

Serum-starved cells were treated for 10 min in 6-well plates with or without 50 μ M homocysteine thiolactone prior to insulin stimulation (100 nM) in Hepes–Ringer buffer containing 10 mM glucose, and 1% bovine serum albumin. Glutathione (250 μ M) was added 5 min before homocysteine addition. After 30 min incubation with insulin, $[\text{U}\text{-}^{14}\text{C}]\text{glucose}$ (1 μ Ci/well) was added and the incubation continued for 1 h. Glycogen was isolated and the radioactivity counted as described previously (Sánchez-Margalet 2000).

RESULTS

Homocysteine thiolactone inhibits insulin-stimulated tyrosine phosphorylation of the insulin-receptor β -subunit

To examine the effect of homocysteine thiolactone on insulin-stimulated tyrosine autophosphorylation of insulin receptors, cells were preincubated with 50 μ M homocysteine thiolactone for 10 min prior to stimulation for 5 min with 100 nM insulin. Cell lysates were subjected to SDS-PAGE and analysed by Western blotting with anti-phosphotyrosine antibodies. As shown in Fig. 1A, insulin-mediated tyrosine phosphorylation of the insulin-receptor

FIGURE 1. Effect of homocysteine thiolactone on insulin-mediated insulin-receptor tyrosine phosphorylation. Serum-starved HTC-IR cells were pretreated with or without 50 μ M homocysteine thiolactone for 10 min before the stimulation with or without 100 nM insulin for 5 min. When employed, glutathione (250 μ M) was added 5 min before the homocysteine. Cell lysates were subjected to SDS-PAGE and analysed by Western blotting with anti-phosphotyrosine (α -PY) (A). Cell lysates were also immunoprecipitated with anti-insulin-receptor β -subunit (α -IR), subjected to SDS-PAGE, and analysed by Western blotting antibody with α -PY (B). IR, insulin-receptor β -subunit; IP, immunoprecipitating antibody; WB, Western blotting antibody. The blots shown are representative of 4 experiments.

β -subunit was prevented by the preincubation with thiolactone. Next, we investigated the possible role of oxidative stress in the inhibitory effect of homocysteine thiolactone. Thus, we added 250 μ M glutathione 5 min before the preincubation with homocysteine thiolactone. As shown in Fig. 1A, glutathione restored the insulin-mediated tyrosine phosphorylation of insulin-receptor β -subunit.

To further demonstrate the effects of homocysteine thiolactone on tyrosine phosphorylation of the insulin-receptor β -subunit, we immunoprecipitated the cell lysates with anti-insulin-receptor β -subunit antibody after the incubation of the cells. Immunoprecipitates were further analyzed by Western blotting with anti-phosphotyrosine antibody. As shown in Fig. 1B, insulin-mediated

tyrosine phosphorylation of the insulin-receptor β -subunit was impaired by the pretreatment of the cells with homocysteine thiolactone. As observed with whole-cell lysates, glutathione completely restored insulin-mediated tyrosine phosphorylation of the insulin-receptor β -subunit, as assessed by analyzing the immunoprecipitates with specific anti-phosphotyrosine immunoblotting. As a control for the amount of insulin receptor, we used a Western blot of the same samples against the insulin-receptor β -subunit.

Inhibition, by homocysteine thiolactone, of insulin-stimulated tyrosine phosphorylation of insulin-receptor substrates

Next, we investigated whether the proximal events in insulin post-receptor signaling were also affected by pretreatment with homocysteine thiolactone, i.e. by tyrosine phosphorylation of the insulin-receptor substrates. We assessed tyrosine phosphorylation of two substrates previously characterized in this hepatoma cell line, IRS-1 and Sam68, one of the p60–70 substrates of the insulin receptor (Sánchez-Margalet 1999, Sánchez-Margalet & Najib 1999), by specific immunoprecipitation followed by anti-phosphotyrosine immunoblotting. As shown in Fig. 2, homocysteine thiolactone pretreatment inhibited the tyrosine phosphorylation of both substrates (IRS-1, Fig. 2A; Sam68, Fig. 2B), in parallel with the observed inhibition on the insulin-receptor β -subunit (Fig. 1). Again, as observed with the anti-insulin receptor immunoprecipitates, glutathione prevented the inhibitory effect of homocysteine thiolactone on the tyrosine phosphorylation of both substrates (IRS-1, Fig. 2A; Sam68, Fig. 2B). The amounts of immunoprecipitated substrate were checked by specific Western blotting of the same samples with either anti-IRS-1 (Fig. 2A), or anti-Sam68 (Fig. 2B).

Homocysteine thiolactone inhibits the association of the insulin-receptor β -subunit and its substrates with p85, the regulatory subunit of PI3K

To clarify the inhibition of insulin-receptor signaling produced by homocysteine thiolactone, we assessed the insulin-mediated association of tyrosine-phosphorylated proteins with p85, the regulatory subunit of PI3K. We had previously characterized the tyrosine-phosphorylated proteins associated with p85 in these cells in response to insulin: the insulin-receptor β -subunit, IRS-1, and p60–70 substrates (Sánchez-Margalet *et al.* 1995, Sánchez-Margalet & Najib 1999). As shown in

FIGURE 2. Effect of homocysteine thiolactone on insulin-induced tyrosine phosphorylation of insulin-receptor substrates. Serum-starved HTC-IR cells were treated as described in the legend to Fig. 1. Solubilized cell lysates were immunoprecipitated with anti-IRS-1 (α -IRS-1) (A), or anti-Sam68 (α -Sam68) (B), subjected to SDS-PAGE, and analysed by Western blotting with α -PY. The same samples were analysed with anti-IRS-1 (A) or anti-Sam68 (B) to check the amount of immunoprecipitated protein. IP, immunoprecipitating antibody; WB, Western blotting antibody. Similar results were obtained in 3 other experiments.

Fig. 3, homocysteine thiolactone impaired the association of the insulin-stimulated tyrosine-phosphorylated proteins with p85, and, again, glutathione prevented this inhibitory effect of homocysteine. The amount of immunoprecipitated p85 was similar in every sample, as assessed using anti-p85 immunoblotting (Fig. 3).

Inhibition by homocysteine thiolactone of phosphatidylinositol 3-kinase activity

Next, we studied the effect of homocysteine thiolactone on PI3K activity. To check whether the thiolactone inhibition of tyrosine phosphorylation of the insulin receptor and substrates and their association with p85-PI3K led to changes in the activity, we measured PI3K activity in anti-phosphotyrosine immunoprecipitates. As shown in Fig. 4, phosphotyrosine-associated PI3K activity

FIGURE 3. Effect of homocysteine thiolactone on the association of tyrosine-phosphorylated proteins with p85, the regulatory subunit of PI3K. Cells were treated as described in the legend to Fig. 1. Solubilized cell lysates were immunoprecipitated with anti-p85 PI3K (α -p85-PIK), subjected to SDS-PAGE, and analysed by Western blotting with α -PY. IR, insulin receptor β -subunit; IRS-1, insulin-receptor substrate-1; p60–70, 60–70 kDa insulin-receptor substrate; IP, immunoprecipitating antibody; WB-AB, Western blotting antibody. The experiment shown is representative of three others.

was stimulated by 100 nM insulin, and 50 μ M homocysteine thiolactone prevented this effect of insulin. In parallel with the phosphorylation data, glutathione (250 μ M) was able to block the inhibitory effect of homocysteine thiolactone on insulin-stimulated PI3K activity (Fig. 4).

Homocysteine thiolactone inhibits insulin-stimulated glycogen synthesis

To investigate the effect of homocysteine thiolactone on the metabolic end-point of the PI3K pathway, i.e. glycogen synthesis (Sánchez-Margalet 2000), we measured glucose incorporation into glycogen, by using [14 C]glucose. As shown in Fig. 5, 50 μ M homocysteine thiolactone completely blocked the metabolic effect of insulin on glycogen synthesis in HTC hepatoma cells. This inhibitory effect of homocysteine thiolactone was fully prevented by the preincubation of cells with glutathione (250 μ M) prior to the addition of homocysteine.

DISCUSSION

In this study, we demonstrate that short-term treatment of HTC-IR cells with micromolar

FIGURE 4. Effect of homocysteine thiolactone on phosphotyrosine-associated PI3K activity phosphatidyl inositol-3-phosphate (PIP3) is the product of PI3K activity. Serum-starved HTC-IR cells were treated as described in the legend to Fig. 1. Solubilized cell lysates were immunoprecipitated with α -PY, and immunoprecipitates were then used to determine PI3K activity. Samples were chromatographed by thin-layer chromatography. The autoradiogram shown is representative of three.

concentrations of homocysteine thiolactone (50 μ M) inhibits insulin signaling, leading to an inhibitory effect on insulin-stimulated glycogen synthesis. Thus, we present data on the inhibition of insulin-receptor kinase, as well as on downstream signaling events such as the tyrosine phosphorylation of insulin-receptor substrates, their association

FIGURE 5. Effect of homocysteine thiolactone on insulin-stimulated glycogen synthesis. Cells were treated as described in the legend for Fig. 1, but insulin (100 nM) stimulation was extended for 30 min. [14 C]Glucose (1 μ Ci/well) was then added to the cells and the incubation continued for 1 h to determine glucose incorporation into glycogen. The data shown are means $\pm$ S.E.M. of four separate experiments.

with p85-PI3K and PI3K activation, one of the major signaling cascades that plays a central role in the regulation of insulin action (Kahn 1994, Cheatham *et al.* 1994, Sánchez-Margalet *et al.* 1994, Sánchez-Margalet 2000). Since the PI3K signaling pathway is dependent on the tyrosine kinase activity of the insulin receptor and the tyrosine phosphorylation of its substrates, the inhibition of PI3K by homocysteine thiolactone may be attributed to defects in the early events of the insulin signaling. A further direct effect of thiolactone on downstream signaling events, such as PI3K activity, cannot be ruled out, however. In fact, the inhibition of PI3K seems stronger than the inhibition observed with tyrosine phosphorylation. Therefore, a multi-step inhibitory effect of homocysteine thiolactone may not be striking. In any case, the major metabolic end-point of the PI3K pathway, i.e. the insulin-stimulated glycogen synthesis (Sánchez-Margalet 2000), is almost completely inhibited by homocysteine thiolactone. Thus, the deleterious effects of homocysteine in insulin signaling translates to an inhibition of insulin action, at least in the context of glycogen synthesis.

Protein homocysteinylation by homocysteine thiolactone, resulting in protein damage, is one of the mechanisms underlying the involvement of homocysteine in the pathology of vascular disease (Jakubowski 1997, 2000). In the light of the present study, such a mechanism is unlikely to mediate the effects of thiolactone on insulin-receptor signaling, since these effects are observed after a short period of treatment. Nevertheless, longer exposures of cells to homocysteine thiolactone might contribute to the protein modification, damage, multimerization and precipitation of molecules of the insulin signaling cascade, leading to a decrease in the amount of functional protein. Consistently, we have not observed any differences in the amounts of signaling proteins under our short-term experimental conditions.

On the other hand, we have observed that glutathione (250 μ M) completely blocked the inhibitory effect of homocysteine thiolactone on insulin signaling and action. Thus, the thiolactone impairment in the autophosphorylation of the insulin receptor, the tyrosine phosphorylation of its substrates, their association with p85-PI3K, and the PI3K activity itself, as well as the insulin-stimulation of glycogen synthesis, are completely restored by glutathione. These results strongly suggest that the mechanism underlying the defects in insulin signaling involves the oxidative stress produced by homocysteine thiolactone. In this context, the inhibition of insulin signaling could be another oxidation-mediated toxic effect on liver cells, in addition to the toxic effects reported for

endothelial cells in culture (de Groot *et al.* 1983, Starkebaum & Harlam 1986, Stamler *et al.* 1993). On the other hand, we have ruled out the possibility that the effect of homocysteine thiolactone on insulin signaling and action is mediated by the inhibition of insulin binding (data not shown). Rather, the effects may be exerted intracellularly – in the insulin-receptor activation and the downstream signaling pathways. In fact, the inhibitory effect of oxidative stress on insulin signaling has been studied previously (Blair *et al.* 1999, Hansen *et al.* 1999, Tirosh *et al.* 1999), as well as the inhibition of insulin action by oxidative stress or the inhibition of glutathione synthesis (Rudich *et al.* 1997, Blair *et al.* 1999, Khamaisi *et al.* 2000). Moreover, infusion of reduced glutathione has been shown to improve insulin sensitivity in type 2 diabetic patients (De Mattia *et al.* 1998).

In summary, these results lead to the conclusion that homocysteine thiolactone inhibits insulin signaling and action by a mechanism involving oxidative stress. Therefore, an effect of homocysteine on insulin resistance *in vivo* might be expected. Thus, homocysteine could be a new factor that functions as a relevant mediator of oxidative stress-induced insulin resistance. This hypothesis remains to be studied, but the data presented here give some clue as to the intracellular mechanisms by which homocysteine may eventually lead to insulin resistance.

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