Homocysteine thiolactone inhibits insulin-stimulated DNA and protein synthesis: possible role of mitogen-activated protein kinase (MAPK), glycogen synthase kinase-3 (GSK-3) and p70 S6K phosphorylation

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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. We have recently found that homocysteine thiolactone inhibits insulin receptor tyrosine kinase activity, which results in decreased phosphatidylinositol 3-kinase (PI3K) activity and inhibition of glycogen synthesis. Oxidative stress seemed to be the mechanism underlying these effects, since glutathione was able to restore the insulin signaling as well as the insulin-mediated glycogen synthesis. In the present work we have further investigated insulin receptor signaling studying mitogen-activated protein kinase (MAPK), glycogen synthase kinase-3 (GSK-3) and p70 S6K phosphorylation. Again, homocysteine thiolactone (50 μ M) prevented insulin-mediated MAPK, GSK-3 and p70 S6K phosphorylation and these effects were blocked by glutathione (250 μ M). Since MAPK and PI3K pathways, including GSK3 and S6K, seem to mediate insulin-mediated growth and proliferation, we measured DNA and protein synthesis. We have found that homocysteine thiolactone (50 μ M) inhibits insulin-mediated growth and proliferation, as previously shown for glycogen synthesis. Again, these effects seem to be mediated by oxidative stress, since 250 μ M glutathione completely abolished the effects of homocysteine thiolactone on insulin-stimulated DNA and protein synthesis. In conclusion, these data suggest that homocysteine thiolactone impairs insulin signaling by a mechanism involving oxidative stress, leading to a defect in the action of insulin on growth and proliferation.

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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 1997). The oxidant stress of hyperhomocysteinemia, 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 supposed 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). Moreover, homocysteine may have a link with insulin resistance; we have previously found that hyperhomocysteinemia is associated with hyperinsulinemia in an oral glucose tolerance test in obese subjects (Sánchez-Margalet *et al.* 2002), and increased plasma homocysteine levels have been observed in the insulin-resistance syndrome (Meigs *et al.* 2001), in patients with polycystic ovary syndrome associated with insulin resistance (Schachter *et al.* 2003) and in women with gestational diabetes mellitus (Seghieri *et al.* 2003). In this context, hyperhomocysteinemia has been proposed as a component of syndrome X (Oron-Herman *et al.* 2003). Oxidative stress

is supposed to mediate the effects of homocysteine on atherosclerosis; however, little is known about the possible effect of homocysteine on insulin signaling. We have recently found that homocysteine thiolactone inhibits insulin signaling in hepatoma cells (HTC) over-expressing human insulin receptors (HTC-IR) (Najib & Sánchez-Margalet 2001), thereby providing some molecular mechanism for the insulin resistance induced by homocysteine. We also found that glutathione had a protective effect on this inhibition. Therefore, we proposed the hypothesis that, *in vitro*, homocysteine thiolactone could inhibit insulin signaling by inducing oxidative stress (Najib & Sánchez-Margalet 2001).

Insulin is known to activate multiple signaling pathways (Cheatham & Kahn 1995, Myers & White 1996, Avruch 1998), but the early 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 Src homology domain 2 (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), or Grb2, which mediates the activation of the Ras-MAPK (mitogen-activated protein kinase) pathway (Skolnik *et al.* 1993). PI3K plays a central role in regulating glucose transport and glycogen synthesis (Sánchez-Margalet *et al.* 1994, Sánchez-Margalet 2000). PI3K pathways can inhibit glycogen synthase kinase-3 (GSK-3) by promoting its phosphorylation (Cross *et al.* 1994, 1995), which results in activation of glycogen synthase. The PI3K pathway also activates p70 S6K, which also mediates glycogen synthesis (Sánchez-Margalet 2000) as well as DNA and protein synthesis (Dixon *et al.* 1999, Vinals *et al.* 1999).

In previous studies we have shown that short-term incubation with homocysteine thiolactone inhibits the early steps of insulin receptor signaling – i.e. tyrosine phosphorylation, protein–protein interaction and PI3K activity – resulting in inhibition of insulin-stimulated glycogen synthesis.

In the present paper we have investigated the insulin receptor signaling pathways downstream of PI3K, by studying phosphorylation levels of GSK-3 and p70 S6K; these not only control glycogen synthesis, but also cell proliferation via accumulation of cytoplasmic β -catenin (Nakamura 1997, Cui *et al.* 1998) and activation of translation machinery (Dixon *et al.* 1999, Vinals *et al.* 1999) respectively. We have also investigated the effect of homocysteine thiolactone on another insulin-stimulated mitogenic pathway, MAPK (Ahn *et al.* 1993, Van Obberghen *et al.* 1993, Cheatham & Kahn 1995, Avruch 1998). Finally, we have studied the inhibitory effect of homocysteine thiolactone on insulin-stimulated growth and proliferation in HTC hepatoma cells.

Materials and methods

Materials

Polyclonal antibodies to phosphorylated GSK-3, which recognize GSK-3 α phosphorylated at serine 21 and GSK-3 β phosphorylated at serine 9, and polyclonal antibodies to phosphorylated p70 S6 kinase (Thr421/Ser424) were obtained from Cell Signaling New England Biolabs (Beverly, MA, USA). Monoclonal antibodies that recognize GSK-3 α and GSK-3 β , and p70 SK were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibodies to phosphorylated p44/42-MAPK (Thr202/Thr204) and monoclonal antibodies that recognize p44/42-MAPK were from New England Biolabs.

Methods

Cells

Rat HTC-IR hepatoma cells were kindly provided by Dr I D Goldfine (UCSF, San Francisco, CA, USA). Cells were prepared and maintained in Dulbecco's modified Eagle's medium (DMEM; 1 g/l glucose) as described previously (Sung *et al.* 1995). For signaling experiments, cells were grown in 100 mm dishes to 90% confluency and were serum-starved for 24 h. They were treated for 10 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. The insulin concentration used is the dose achieving maximal effects in insulin receptor signaling (Sung *et al.* 1994). The concentration of homocysteine thiolactone used in the experiments was the lowest concentration to achieve maximal inhibition of insulin receptor activation (Najib & Sánchez-Margalet 2001) and is a concentration that can be achieved in intermediate hyperhomocysteinemia (15–50 μ M). In some experiments, 250 μ M glutathione was added 5 min before homocysteine thiolactone preincubation. This concentration of glutathione was previously found to prevent the inhibitory effect of homocysteine in insulin receptor activation (Najib & Sánchez-Margalet 2001). Homocysteine and glutathione were not washed before insulin stimulation, and were present during the incubation time. 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 MgCl₂, 1 mM CaCl₂, 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. For insulin-action experiments, cells were grown in six-well plates to 90% confluence and were serum-starved for 16 h.

Figure 1 Effect of homocysteine thiolactone on insulin-mediated phosphorylation of GSK-3. Serum-starved HTC-IR cells were pretreated with or without 50 μ M homocysteine thiolactone for 10 min before stimulation with or without 100 nM insulin for 10 min. When employed, glutathione (250 μ M) was added 5 min before homocysteine. Cell lysates were subjected to SDS-PAGE and analysed by western blotting with antibodies that recognize GSK-3 phosphorylated at serine 21 (GSK-3 α) and 9 (GSK-3 β). Cell lysates were also analysed by immunoblot with anti-GSK-3. Blots are representative experiments out of four.

Phosphorylation of GSK-3, p70 S6K and MAPK

Activation or inactivation of GSK-3, p70 S6K and MAPK were evaluated by the phosphorylation status, employing antibodies that specifically recognize the phosphorylated form of the proteins (New England BioLabs).

Cells were solubilized for 30 min at 4 °C in lysis buffer as previously described (Sung *et al.* 1994). Protein concentration was determined using the Micro BCA protein assay kit from Pierce (Rockford, IL, USA). Samples were denatured and resolved by SDS-PAGE, transferred onto nitrocellulose membranes and analyzed by immunoblot using the appropriate antibodies as previously described (Sung *et al.* 1994, Sánchez-Margalet & Najib 1999) using a highly sensitive chemiluminescence system (Supersignal, Pierce). The amount of GSK-3, p70 S6K and p42/44 MAPK in each lane was controlled by immunoblot with the corresponding antibodies, which were employed to normalize the results. The bands obtained were scanned and analyzed using the PCBAS2.0 program (Raytest Isotopenmessgeräte, Straubenhardt, Germany).

[³H]Thymidine incorporation into DNA

Cells were treated for 6 h with different stimuli. Next, [³H]thymidine (0.5 μ Ci/well) was added and incubation continued for 4 h. Cells were washed with PBS and solubilized in 0.03% SDS, precipitated with 10% trichloroacetic acid (TCA) and radioactivity measured by a liquid scintillation counter as previously described (Sánchez-Margalet *et al.* 1994).

[³H]Leucine incorporation into protein

Cells were treated for 3 h with or without different stimuli. Next, L-[2,3,4,5-³H]leucine (0.5 μ Ci/well) was

added and incubation continued for 2 h. Cells were then washed and solubilized in 0.03% SDS. Samples were then TCA precipitated and counted as described previously (Sánchez-Margalet *et al.* 1994).

Statistical analysis

Values presented are means $\pm$ S.E.M. The statistical study was performed by ANOVA and Student's *t*-test for paired data; Bonferroni *post-hoc* test was used to compare values from different concentration points. Differences were considered significant when $P < 0.05$.

Results

Homocysteine thiolactone inhibits insulin-stimulated GSK-3 and p70 S6K phosphorylation

To examine the effect of homocysteine thiolactone on insulin-stimulated GSK-3 inhibition, we studied the phosphorylation level of GSK-3 using specific antibodies that recognize GSK-3 phosphorylated at serine 21 (GSK-3 α) and 9 (GSK-3 β). GSK-3 is one of the known targets of protein kinase B (PKB), and is the mechanism whereby insulin inactivates this enzyme (van Weeren *et al.* 1998). Serum-starved HTC-IR cells were preincubated with 50 μ M homocysteine thiolactone for 10 min prior to stimulation for 10 min with 100 nM insulin. Cell lysates were subjected to SDS-PAGE and were analysed by western blotting with antibodies against the phosphorylated forms of GSK-3. As shown in Fig. 1, insulin-mediated phosphorylation of both isoforms, GSK- α and GSK- β , was prevented by preincubation with homocysteine thiolactone. We next investigated the possible role of oxidative stress in the inhibitory effect

Figure 2 Effect of homocysteine thiolactone on insulin-mediated phosphorylation of S6K. Cells were treated as described in the legend for Fig. 1. Cell lysates were subjected to SDS-PAGE and analysed by immunoblot with anti-phospho-S6K. Results are representative of three experiments.

of homocysteine thiolactone. Thus, we added 250 μ M glutathione 5 min before the preincubation of HTC-IR cells with homocysteine thiolactone. As shown in Fig. 1, glutathione restored the insulin-mediated phosphorylation of both isoforms (GSK-3 α and GSK-3 β).

Downstream of PI3K and 3-phosphoinositide-dependent protein kinase (PDK) in insulin receptor signaling, p70 S6K is also activated. Since p70 S6K activity is correlated with its phosphorylation state, specifically Thr421 and Ser424 (Alessi *et al.* 1998, Pullen *et al.* 1998), we employed polyclonal antibodies that detect p70 S6K only when phosphorylated at Thr421/Ser424. Therefore, we analyzed the same cell lysates used in Fig. 1 by immunoblot with anti-phospho-S6K antibodies. As shown in Fig. 2, we observed results parallel to those of GSK-3. Thus, insulin stimulated the phosphorylation of p70 S6K, and this effect was inhibited by preincubation with homocysteine thiolactone. Again, preincubation of cells with 250 μ M glutathione prevented the inhibitory effect of homocysteine thiolactone on insulin-mediated p70 S6K phosphorylation.

Homocysteine thiolactone inhibits insulin-mediated activation of the MAPK pathway

In order to explore the MAPK pathway, we investigated the threonine phosphorylation level of MAPK (Thr202/Thr204) because MAPK kinase (MEK), the upstream kinase, is known to activate MAPK through phosphorylation of threonine and tyrosine residues (Payne *et al.* 1991), which is the mechanism of activation in response to insulin. Thus, we employed antibodies that specifically recognize the phosphorylated form of p44/42 MAPK to assess the activation of this pathway. As shown in Fig. 3, insulin strongly stimulated the phosphorylation of both p44 and p42 MAPK. However,

when cells were preincubated for 10 min in the presence of 50 μ M homocysteine thiolactone, before insulin stimulation, the effect was abolished. We also wanted to investigate the possible role of oxidative stress in the homocysteine inhibition of the insulin-stimulated MAPK pathway. Thus, we added 250 mM glutathione 5 min before the preincubation with homocysteine thiolactone, and the effect of insulin on p44/42 MAPK phosphorylation was restored (Fig. 3).

Homocysteine thiolactone inhibits insulin-mediated stimulation of growth and proliferation

Since GSK-3, S6K and MAPK pathways regulate growth and proliferation, we investigated the effect of homocysteine in these insulin-mediated effects. As shown in Fig. 4, 50 μ M homocysteine thiolactone completely blocked the mitogenic effect of insulin in HTC-IR 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, suggesting a possible role of oxidative stress in the inhibitory effect of homocysteine.

We also studied the effect of homocysteine on insulin-stimulated protein synthesis. As shown in Fig. 5, 50 μ M homocysteine thiolactone completely blocked the effect of insulin stimulating protein synthesis in HTC-IR hepatoma cells. Again, 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

We have recently found that homocysteine thiolactone inhibits insulin receptor tyrosine kinase, phosphorylation

Figure 3 Effect of homocysteine thiolactone on insulin-mediated p42/44 MAPK phosphorylation. Cells were treated as described in the legend for Fig. 1. When employed, glutathione (250 μM) was added 5 min before homocysteine. Cell lysates were subjected to SDS–PAGE and analysed by western blotting with anti-phospho-MAPK. Cell lysates were also analysed by western blotting with anti-MAPK antibody. Blots are representative experiments out of four.

of insulin receptor substrates and insulin-stimulated PI3K activity, leading to an inhibitory effect on insulin-mediated glycogen synthesis in HTC hepatoma cells overexpressing human insulin receptor (HTC-IR) (Najib & Sánchez-Margalet 2001). In the present study we demonstrate that short-term treatment of HTC-IR cells with micromolar concentrations of homocysteine thiolactone (50 μM) also inhibits mitogenic insulin

signaling and action, leading to an inhibitory effect on insulin-stimulated DNA and protein synthesis.

First, we present data showing homocysteine inhibition of insulin-stimulated mitotic signaling pathways – GSK-3, p70 S6K and MAPK – which are known to mediate signals of cell growth and proliferation (Plyte *et al.* 1992, Ahn *et al.* 1993, Van Obberghen *et al.* 1993, Cheatham & Kahn 1995, Avruch 1998, Cui *et al.* 1998,

Figure 4 Homocysteine thiolactone inhibits DNA synthesis and the protective effect of glutathione. Cells were treated as described in the legend for Fig. 1, but insulin (100 nM) stimulation was extended for 6 h. Next, [³H]thymidine (0.5 μCi/well) was added and incubation continued for 4 h. Cells were washed with PBS and solubilized in 0.03% SDS, precipitated with 10% TCA and radioactivity was measured by a liquid scintillation counter. When employed, glutathione (250 μM) was added 5 min before homocysteine. Data are means±S.E.M. of four separate experiments. **P*<0.05 compared with basal.

Figure 5 Homocysteine thiolactone inhibits protein synthesis and the protective effect of glutathione. Cells were treated as described in the legend for Fig. 1, but insulin (100 nM) stimulation was extended for 3 h, and then [³H]leucine (0.5 μCi) was added and incubation continued for 2 h. Cells were then washed, solubilized and TCA precipitated. Radioactivity was finally measured by a liquid scintillation counter. When employed, glutathione (250 μM) was added 5 min before homocysteine. Data are means±S.E.M. of four separate experiments performed in triplicate. **P*<0.05 compared with basal.

Dixon *et al.* 1999, Vinals *et al.* 1999). Since we have previously observed the inhibitory effect of homocysteine thiolactone in early events of insulin receptor signaling – i.e. the tyrosine phosphorylation of substrates and the interaction with p85 PI3K and PI3K activity (Najib & Sánchez-Margalet 2001) – the inhibition of GSK-3 phosphorylation may be secondary to the inhibition of the PI3K pathway because GSK-3 is a target of PKB (Cross *et al.* 1995), which is downstream of PI3K (Cheatham & Kahn 1995, Sánchez-Margalet 2000). In fact, the inhibition of GSK-3 phosphorylation by homocysteine may also explain the inhibitory effect on insulin-mediated glycogen synthesis previously observed in HTC-IR cells (Najib & Sánchez-Margalet 2001). However, GSK-3 phosphorylates a broad range of substrates in addition to glycogen synthase, including important growth regulators such as β -catenin (Ikeda *et al.* 1998), cyclin D1 (Diehl *et al.* 1998), or p21 (Rossig *et al.* 2002), as well as numerous transcription factors (Manoukian & Woodgett 2002). Therefore the effect of homocysteine thiolactone inhibiting growth and proliferation may be partially mediated by preventing GSK-3 phosphorylation. In support of this, insulin-stimulated PKB activity has been previously found to be prevented by oxidative stress (Pham *et al.* 2000). Moreover, the PI3K pathway also mediates the activation of p70 S6K, by phosphorylation via PDK or mammalian target of rapamycin (mTOR) (Sehgal 1995, Alessi *et al.* 1998, Pullen *et al.* 1998). mTOR and S6K may also mediate insulin-stimulated glycogen synthesis (Sánchez-Margalet 2000), as well as DNA and protein synthesis (Dixon *et al.* 1999, Vinals *et al.* 1999). Therefore, inhibition by homocysteine thiolactone of insulin-mediated p70 S6K may partly mediate the inhibitory effect of homocysteine on glycogen synthesis in these cells (Najib & Sánchez-Margalet 2001), as well as the inhibition of protein synthesis that we have observed in the present work.

On the other hand, we have previously observed homocysteine inhibition in the tyrosine phosphorylation of insulin receptor substrates, resulting in the inhibition of their interaction with p85 PI3K (Najib & Sánchez-Margalet 2001). Similarly, the inhibition of tyrosine phosphorylation may also impair their interaction with Grb2-SOS, inhibiting the Ras signaling pathway. In this way, homocysteine thiolactone incubation may finally lead to the inhibition of MAPK, as we have assessed in the present work. However, we have not checked the effects on the early steps of the MAPK pathway.

However, we do not know whether other effects on the MAPK pathway, such as the activation of MAPK phosphatases (MKP), may be contributing to the inhibition of MAPK phosphorylation (Bhalla *et al.* 2002). In this context, oxidative stress is known to induce the expression and activity of MKP-1 (Ahkand *et al.* 2001, Xu *et al.* 2004). Since the homocysteine inhibition of MAPK phosphorylation is reversed by glutathione, part

of the effect could be mediated by the oxidative stress on some MKP. The effect of homocysteine would be more likely to be due to the regulation of the activity, rather than the induction of expression, because it is observed in short-term incubation. This is a speculation that may be worth investigating, however. In any case, the homocysteine inhibition of the tyrosine phosphorylation of insulin receptor substrates may be sufficient for the impairment of the MAPK pathway, since it depends on tyrosine kinase activity (Lipson *et al.* 1998).

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, Najib & Sánchez-Margalet 2001), 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, Najib & Sánchez-Margalet 2001). Regarding the action of insulin on DNA synthesis and proliferation, oxidative stress has been demonstrated to inhibit this specific insulin effect (Orzechowski *et al.* 2002), which is restored with anti-oxidant agents. Similarly, other groups have shown that oxidative stress also prevents insulin-stimulated protein synthesis by inhibiting PKB and 4 EBP1 (Pham *et al.* 2000). Our data show homocysteine inhibition of insulin-mediated GSK, p70 S6K and MAPK pathways – resulting in an impairment in insulin action, i.e. growth and proliferation – by a mechanism mediated by oxidative stress; therefore, our data are consistent with previous work by other groups and provide new evidence to support the hypothesis of oxidative stress as a cause of insulin resistance. In this context, the inhibition of insulin signaling and insulin action could be another oxidation-mediated toxic effect of homocysteine on liver cells, in addition to the reported toxic effects in endothelial cells in culture (de Groot *et al.* 1983, Starkebaum & Harlam 1986, Stamler *et al.* 1993). Therefore, our results further implicate homocysteine in the pathophysiology of oxidative stress and point to a possible role of homocysteine in insulin resistance.

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