Garlic Extract Methylallyl Thiosulfinate Blocks Insulin Potentiation of Platelet-Derived Growth Factor–Stimulated Migration of Vascular Smooth Muscle Cells

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Platelet-derived growth factor (PDGF) is a potent inducer of vascular smooth muscle cell (VSMC) migration, whereas insulin, in physiological concentrations, helps maintain the nonproliferative phenotype of these cells. However, hyperinsulinemia (10 nmol/L) significantly potentiates the PDGF (30 pmol/L)-induced migration of VSMC. This potentiating effect of hyperinsulinemia appears to be mediated by increased availability of geranylgeranylated Rho-A. Hyperinsulinemia significantly increased the activity of geranylgeranyltransferase I (GGTase I) and the amounts of prenylated Rho-A. This action of hyperinsulinemia was inhibited by methylallyl thiosulfinate (MAT), a component of garlic extract, which exerted a strong anti-GGTase I activity. MAT also completely inhibited the ability of hyperinsulinemia to potentiate the PDGF-induced VSMC migration. Thus, the purported anti-atherogenic action of garlic may be related to its inhibitory influence on GGTase I.

AN IMPORTANT FEATURE of atherosclerosis is the conversion of quiescent or differentiated vascular smooth muscle cells (VSMC) into proliferative or de-differentiated ones, resulting in enhanced migration of VSMC that underscores this change in VSMC phenotype.[1] While the mechanism of the phenotypical modulation of VSMC remains incompletely understood, platelet-derived growth factor (PDGF) is known to be one of the major stimuli promoting migration and proliferation of VSMC.[2] Furthermore, while ambient normoinisulinemia helps maintain the differentiated phenotype of VSMC,[3] previously reported observations indicate that hyperinsulinemia may potentiate the stimulatory effects of PDGF on DNA synthesis, amounts of vascular endothelial growth factor (VEGF) mRNA,[4] and VSMC migration.[5] The ability of hyperinsulinemia to promote prenylation of Ras and Rho proteins has been proposed as an underlying mechanism of the potentiating action of insulin.[4-7]

Insulin has been shown to stimulate the phosphorylation of the α-subunit of farnesyltransferase (FTase) and geranylgeranyltransferase I (GGTase I).[8,9] These 2 prenyltransferases share the same α-subunit, while the β-subunit confers substrate specificity,[10] governing either farnesylation or geranylgeranylation of small molecular weight guanosine triphosphatases (GTPases). Increased phosphorylation of the α-subunit is accompanied by augmented activity of these 2 ubiquitous enzymes that prenylate Ras and Rho proteins, respectively.[8,9] Prenylation of these proteins is a prerequisite for their subsequent activation by various growth factors.[11]

Because activation of Rho proteins appears to be critical for the cytoskeletal changes responsible for cell migration,[12, 13] we examined whether hyperinsulinemia-induced potentiation of PDGF action can be blocked by interference with activity of GGTase I. Moreover, because major active components of garlic, thiosulfinates, have been shown to possess a strong anti-GGTase I activity, we assessed the influence of methylallyl thiosulfinate (MAT) on the ability of insulin to stimulate GGTase I, increase the amounts of geranylgeranylated Rho-A, and potentiate PDGF action in VSMC.

MATERIALS AND METHODS

All standard chemicals were from Sigma (St Louis, MO). Anti–Rho-A antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-mitogen-activated protein kinase (MAPK) and anti-phospho-Akt were from Cell Signaling (Beverly, MA). All supplies and reagents for sodium dodecyl sulfate polyarcrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad (Hercules, CA), and the enhanced chemiluminescence kit was from AmershamBiosciences (Piscataway, NJ). Cell culture media and supplies were from Life Technologies. Gemini Bio-Products (Rockville, MD), Promega (Madison, WI), Calbiochem (San Diego, CA), and Sigma. Radioisotopes were from DuPont New England Nuclear (Boston, MA). Insulin was from Eli Lilly (Indianapolis, IN). Ras-CVLL, GGTI-286, and *Clostridium botulinum* C3 transferase were from Calbiochem. Methylallyl thiosulfinate was isolated in the laboratory of one of the authors (Y.Y.), while a dominant negative constract of Rho-A (threonine is substituted for asparagine at position 19) was a gift from Dr J. Baltassare (St Louis University, St Louis, MO).

Isolation, Characterization, and Growth of VSMC

Bovine aortic smooth muscle cells were routinely isolated and grown from explants of small pieces of aortic tissue from adult animals. Cells were passaged from explant cultures into 75-cm^2 tissue culture flasks after trypsinization. Cells were then cultured in growth medium, consisting of minimal essential medium (MEM) and containing 1x nonessential amino acids, 5 mmol/L glucose, 0.4 mmol/L glutamine, penicillin/streptomycin, and 10% fetal bovine serum (FBS) (Gemini Bio-Products) Passages 1 through 4 were used in these experiments.

Migration of VSMC

For studying migration properties of VSMC, we used 24-well microchemotaxis chambers with polycarbonate membranes (Costar, Corning, NY). Polycarbonate membranes were coated with collagen (Type I Collagen, Worthington Biochemical, Lakewood, NJ, 60 μg/mL). Subconfluent (60% to 70%) serum-starved VSMC were treated with insulin alone or in the presence of other agents as indicated in the

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text for 24 hours, trypsinized, and resuspended in serum-free medium. Cells were counted in a hemacytometer. A suspension of 30,000 VSMC was added to the upper chamber of each well. After 15 minutes, PDGF (30 pmol/L) was placed in the lower chamber of designated wells. VSMC were incubated at 37°C in 5% CO_2, 95% O_2 for 1.5 hours. Membranes were taken out of the wells, fixed, and stained (Diff-Quick Stain Set, Dade Behring, Newark, NJ).

VSMC that had not migrated through the membrane were removed from the upper side of the membrane with a wet cotton swab. Membranes with migrated VSMC were attached to glass slides and counted in 8 viewing fields per experiment using a light microscope.

Digital Deconvolution Microscopy

Cultured bovine aortic smooth muscle cells were trypsinized and re-suspended in MEM containing 10% FBS. The cells were counted using hemacytometer and plated onto sterile glass cover slips (FISHER, Hanover Park, IL, 12 mm in diameter) in a 24-well plate (1.5×10^4 cells per each well). For the next 24 hours, cells were incubated in serum-free MEM at 37°C under 5% CO_2 and then treated with insulin (10 nmol/L), garlic extract, diallyl thiosulfinate (40 μmol/L), and GGTI-286 (3 μmol/L) for 24 hours and PDGF (30 pmol/L) for 1.5 hours. After incubation, cells were fixed with 4% paraformaldehyde/phosphate-buffered solution (PBS) solution and permeabilized with Triton X-100 for 30 minutes. For F-actin staining purposes, VSMC were incubated with rhodamine phalloidin (Molecular Probes, Eugene, OR) for 1 hour and the cover slips mounted to glass slides using 0.1% *p*-phenylenediamine in 75% glycerol/PBS.

Fluorescent images were captured using a 40x oil immersion objective on a Zeiss Axioplan 2 microscope equipped with a Cooke sensicam deep-cooled charged coupled device (CCD) camera and Slidebook software analysis program (Intelligent Imaging Innovations, Denver, CO). Lamellipodia were counted using 40x air objective.

In Vitro GGTase I Activity Assay

VSMC were challenged with insulin (10 nmol/L) for 30 and 60 minutes and 24 hours and then lysed in buffer (150 mmol/L NaCl, 5 mmol/L, $MgCl_2$, 1 mmol/L phenylmethylsulfonlyl gluoride, 1 mmol/L dithiothreitol, 1 mmol/L sodium vanadate, 1 mmol/L sodium phosphate, 1% Triton X-100, 0.05% SDS, 1.5 μmol/L aprotinin, 22 μmol/L leupeptin, 50 mmol/L HEPES, pH 7.5). GGTase I activity was assayed in vitro using a modified version of a method by Moores et al.[14] Briefly, lysates containing endogenous GGTase I from control and insulin-treated VSMCs were incubated with 100 nmol/L Ras-CVLL protein and 100 nmol/L tritiated geranylgeranyl pyrophosphate ([^{3}H]GGPP) (15 mCi/mmol at 37°C for 30 minutes). The assay was stopped with 1 mL of 1 mol/L HCl in ethanol, filtered through Whatman (Maidstone, England) GF/C glass-fiber filters, and air-dried. Labeled protein was quantified by liquid scintillation spectrometry.

Separation of Prenylated and Unprenylated Rho-A

VSMC were incubated in serum-free medium with or without insulin (10 nmol/L) for 24 hours and lysed. Lysates were mixed with an equal volume of 4% Triton X-114 and incubated at 37°C for 3 minutes. Aqueous and detergent phases were allowed to separate at room temperature, as previously described.[4-7] The amount of prenylated Rho-A protein was expressed as a percentage of total cellular Rho-A immunoprecipitated from both phases.

Phosphorylation of MAPK and Akt

VSMC were challenged with insulin (10 nmol/L) or PDGF (30 nmol/L) in the absence or presence of 40 μmol/L MAT for 5 or 15 minutes. Cell lysates were normalized for protein, resolved by SDS-PAGE, and immunoblotted with either anti-phospho-MAPK or anti-phospho-Akt antibodies. Phosphorylated MAPK and Akt were determined by chemiluminescence and quantified by densitometry.

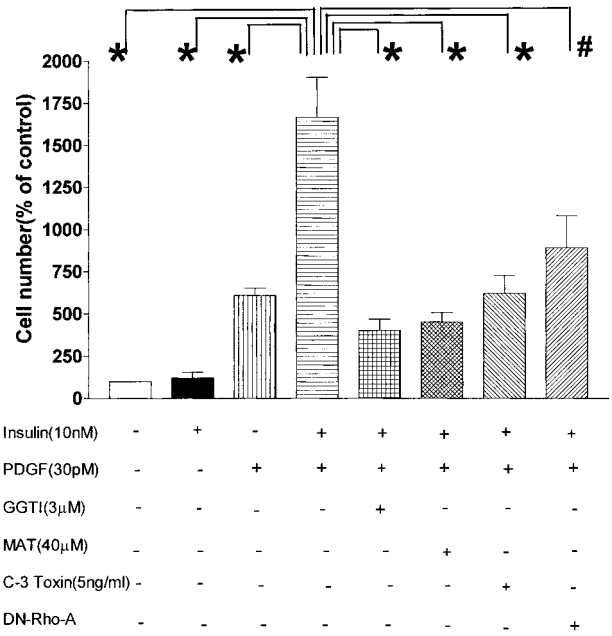


Fig 1. Migration of VSMC. Migration assay was performed as described in the Methods. Subconfluent VSMC were treated with either insulin (10 nmol/L) or PDGF (30 pmol/L). Designated wells were pretreated with insulin alone for 24 h or with insulin and other agents, as indicated, prior to a challenge with PDGF. Results represent the Mean ± SEM of 4 independent experiments. *$P < .001$; #$P < .05$.

Statistical Analysis

Data were analyzed using Student's *t* test, with a *P* value less than .05 considered statistically significant.

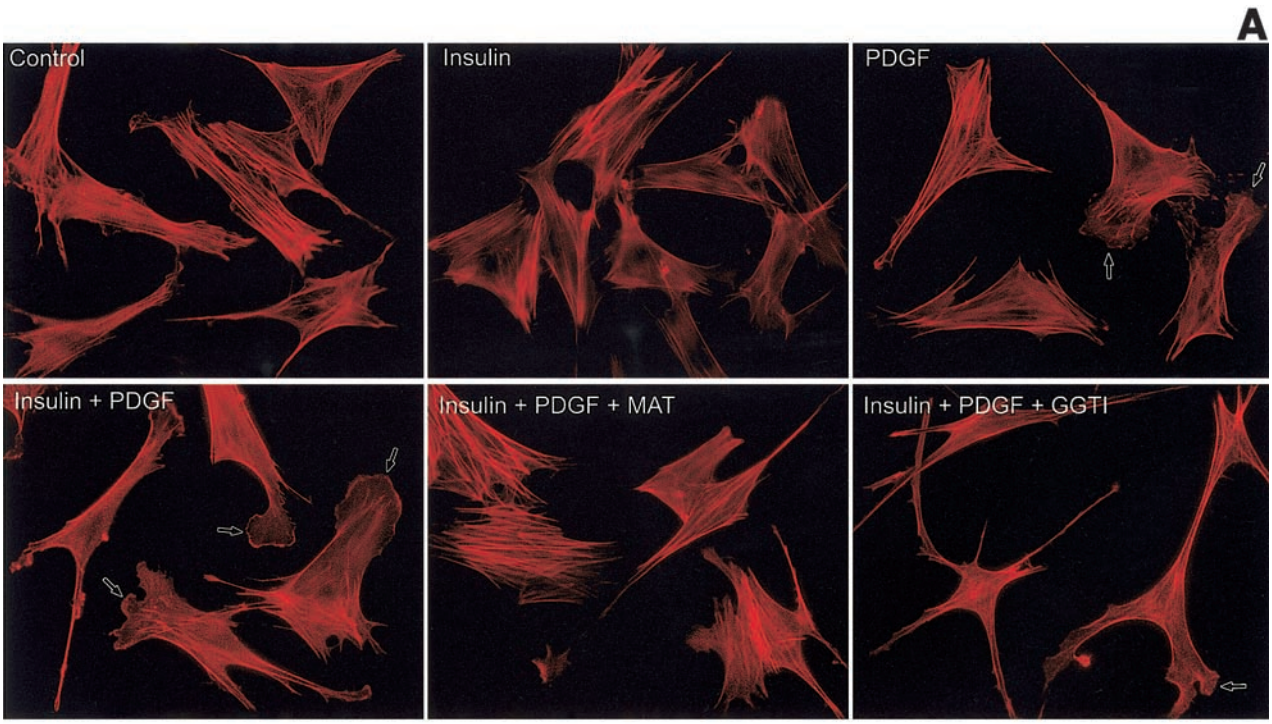
RESULTS

We initially determined whether hyperinsulinemia modulated the effect of PDGF on migration of VSMC. In order to observe a possible stimulatory influence of hyperinsulinemia, we used submaximally effective doses of PDGF (30 pmol/L). PDGF alone significantly increased migration of VSMC, whereas insulin was without effect (Fig 1). However, preincubation of VSMC with insulin (10 nmol/L) for 24 hours resulted in significant augmentation of the effect of PDGF on cell migration.

Because we[4] and others[5] have previously shown that this potentiating influence of hyperinsulinemia is mediated by increased prenylation of Rho-A, we examined whether the effects of hyperinsulinemia on the PDGF-stimulated cell migration were also dependent on prenylation of Rho-A. We used 3 different approaches to address this question (Fig 1). First, we demonstrated that GGTI-286, an inhibitor of GGTase I, which prenylates Rho-A, blocked the potentiating effect of hyperinsulinemia. Second, the inhibition of Rho-A activity by botulinum C-3 toxin, which adenosine diphosphate (ADP) ribosylates and blocks Rho activity, had a similar inhibitory effect. Finally, we observed that transfection of VSMC with a dominant negative mutant of Rho-A also blocked the potentiating effect of insulin (Fig 1).

Because recent studies have demonstrated a strong inhibitory effect of the garlic extract, MAT, on GGTase I,[15] we used this agent to examine its effectiveness in inhibiting the potentiating influence of hyperinsulinemia on the PDGF-stimulated VSMC migration. MAT had a robust inhibitory effect and completely blocked the potentiating effect of insulin on the PDGF-stimulated VSMC migration (Fig 1). MAT was as effective as GGTI-286.

To further quantitate the effects of various agents on VSMC migration, we used digital deconvolution microscopy to analyze actin arrangements and formation of lamellipodia, structural elements reflecting the migratory phenotype of VSMC. PDGF significantly stimulated the formation of lamellipodia and preincubations of cells with insulin (10 nmol/L) significantly augmented this effect of PDGF (Fig 2). Both an inhibitor of GGTase I, GGTI-286, and MAT disrupted actin filaments



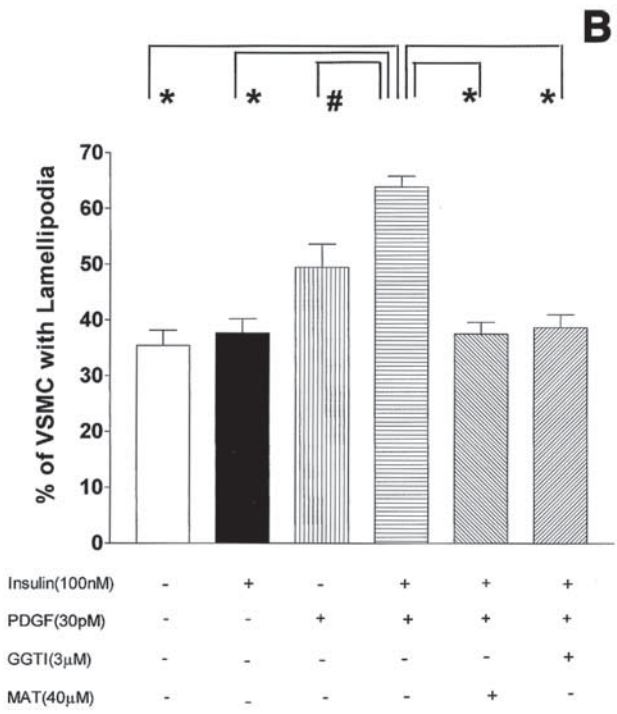


Fig 2. Formation of lamellipodia in VSMC. Cells were treated with insulin, PDGF and various agents as described in the text and in the legend to Fig 1. (A) Representative cells from wells with different treatment (arrows point at lamellipodia). (B) Results of 5 independent experiments (mean ± SEM). *$P < .01$; #$P < .05$.

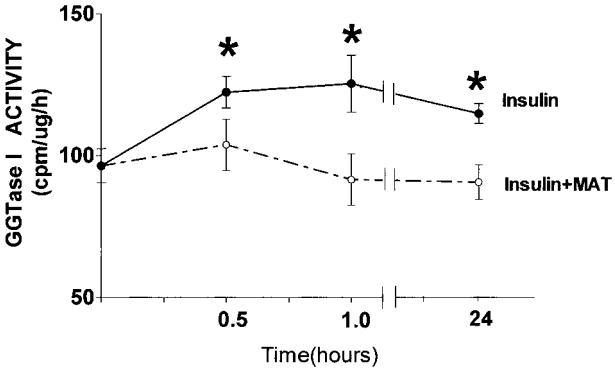


Fig 3. Inhibition of GGTase I activity by MAT. Cells were incubated either with insulin alone (10 nmol/L) or with insulin (10 nmol/L) and MAT (50 μmol/L) for indicated periods of time. Results represent the mean ± SEM of 4 independent experiments. *$P < .05$.

and completely blocked formation of lamellipodia, with MAT being as potent as GGTI-286.

Because the potentiating effects of insulin are mediated by its stimulatory action on GGTase I[4-7] and because MAT has been shown to exert an anti–GGTase I effect,[15] we examined directly the ability of MAT to inhibit GGTase I activity in response to hyperinsulinemia. As expected, insulin activated GGTase I in the absence of MAT. However, MAT completely blocked this effect of insulin (Fig 3).

Increased activity of GGTase I results in increased amounts of geranylgeranylated Rho-A. Accordingly, insulin significantly increased the amounts of geranylgeranylated Rho-A in VSMC, and this effect of insulin was also significantly inhibited by MAT (Fig 4).

In addition to its inhibitory action on GGTase I, MAT could also interfere with either insulin or PDGF signaling and thus block their effects on VSMC migration. To rule out the potential influence of MAT on either insulin or PDGF signal transduction, we assessed its effects on activation of MAP kinase and Akt in VSMC challenged with insulin or PDGF. Both insulin and PDGF stimulated the phosphorylation of these 2 kinases and MAT had no effect on this stimulation at 5 (Fig 5) or 15 minutes (not shown), indicating that MAT does not interfere with the signaling cascade of either hormone.

DISCUSSION

The present data clearly demonstrate three salient points. First, we confirm that hyperinsulinemia potentiates the effect of PDGF on migration of VSMC. Second, we show that inhibition of the ability of hyperinsulinemia to activate GGTase I blocks the potentiating influence of hyperinsulinemia on the PDGF-induced migration of VSMC. Third, our data demonstrate for the first time that garlic extract, MAT, is as effective as a known inhibitor of GGTase I, GGTI-286, in blocking the potentiating influence of hyperinsulinemia.

The low molecular weight Rho-A protein belongs to a larger family of small GTPases that includes Rac, Cdc 42, TC 10, and other Rho proteins.[16] These proteins act as molecular switches to modulate functions of the actin cytoskeleton.[12,13] In differentiated VSMC, Rho is also a mediator of Ca^{2+} sensitization,

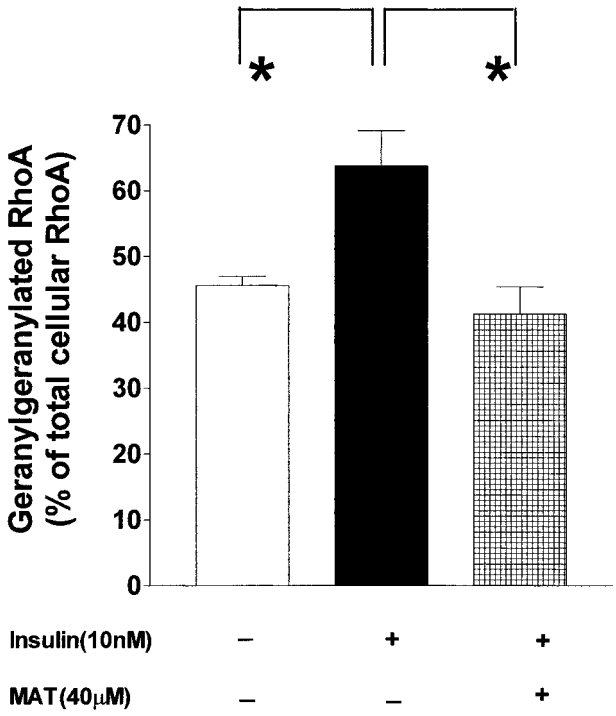


Fig 4. Inhibition of prenylation of Rho-A by MAT. Cells were incubated either with insulin alone (10 nmol/L) or with insulin and MAT (50 μmol/L) for 24 hours. Amounts of geranylgeranylated Rho-A were determined as described in the Methods. Results represent the mean ± SEM of 5 independent experiments. *$P < .01$.

thus participating in the mechanism of VSMC contractility.[17] On the other hand, Rho proteins have been shown to mediate thrombin effects on DNA synthesis and migration in rat aortic VSMC.[18] Our observations are in agreement with the proposed

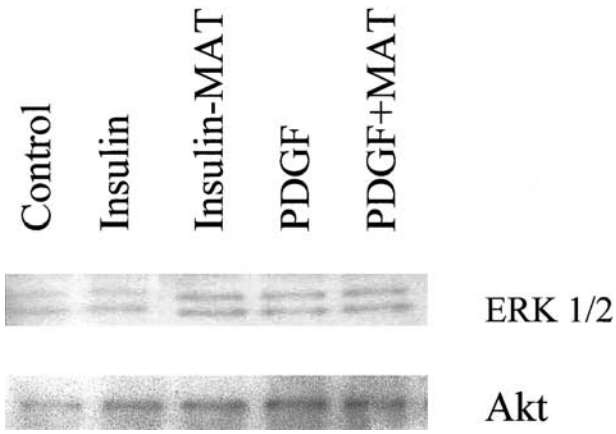


Fig 5. Effect of allicin on insulin and PDGF-stimulated phosphorylation of MAPK Akt. VSMC were challenged with either insulin (10 nmol/L) or PDGF (30 pmol/L) in the absence or in the presence of MAT (50 μmol/L). Phospho-MAPK (ERK 1 and 2) and phospho-Akt were detected by Western blot. A representative experiment (of 3 performed) demonstrates no effect of MAT on either insulin- or PDGF-stimulated phosphorylation of these 2 kinases.

role of Rho proteins in the mechanism of VSMC migration. Moreover, we demonstrated that inhibition of Rho activation by the inhibitors of GGTase I (GGTI-286 and MAT), by ADP-ribosylation (C-3 toxin), or by an introduction of a dominant negative mutant of Rho-A blocked the ability of hyperinsulinemia to potentiate the effect of PDGF (Fig 1).

Geranylgeranylation of Rho proteins is a prerequisite for their subsequent activation by GTP loading under the influence of several growth factors.[19] Geranylgeranylation of Rho-A is governed by the enzyme GGTase I.[11] We have recently demonstrated that insulin promotes the phosphorylation its α-subunit and activation of this enzyme in VSMC and MCF-7 breast cancer cells.[5,6,20] Under the influence of hyperinsulinemia, these cells have increased the amounts of prenylated Rho-A and display exaggerated mitogenic responsiveness to other growth factors.[6,7,20] In VSMC, hyperinsulinemia in a dose-dependent manner significantly enhanced the influence of angiotensin II, hyperglycemia, and advanced glycosylation end products on nuclear factor-κB–dependent transcriptional activity,[20] while in MCF-7 cells, hyperinsulinemia potentiated nuclear effects of lysophosphatidic acid.[6,21] These potentiating effects of hyperinsulinemia were completely blocked by GGTI-286, C-3 toxin, and by a dominant negative Rho-A.[6,20,21] Thus, we postulated that hyperinsulinemia primes various tissues to the mitogenic actions of other growth factors via its ability to significantly augment the prenylation of small molecular weight GTPases.[4-7] Inhibitors of the prenyltransferases completely block this modulatory effect of hyperinsulinemia. In VSMC stimulatory effects of hyperinsulinemia on geranylgeranylation of Rho-A appear to be responsible for the potentiating influence of insulin on the PDGF-induced VSMC migration. The role of the insulin-stimulated prenylation of Rac or Cdc 42 in the mechanism governing migration of VSMC remains to be elucidated.

Hyperinsulinemia is a hallmark of insulin resistance, arising as a compensatory step in response to an impairment in the metabolic action of insulin. At the level of insulin signaling, this impairment(s) lies along the insulin receptor substrate-phopshatidylinositol 3-kinase pathway, whereas activation of the Shc-Ras-MAP kinase-dependent pathway remains unaffected.[22–24] Insulin action on the prenyltransferases is also unaffected by the metabolic insulin resistance.[8,9,24] Thus, compensatory hyperinsulinemia associated with the metabolic insulin resistance continues to potentiate mitogenic activities of various growth factors, including PDGF, insulin-like growth factor-1 (IGF-1), endothelial growth factor (EGF), angiotensin II, and hyperglycemia, by increasing the amounts of prenylated Ras and Rho proteins available for activation.[4,6,7,20]

Garlic has been used as a spice and folk medicine since ancient times. In modern times, several groups of investigators have reported its medicinal, antimicrobial, and antitumor properties.[25,26] Singh et al[27] recently reported that MAT treatment significantly inhibited the growth of H-*ras* oncogene-transformed tumors in nude mice.[27,28] They suggested that MAT inhibited the membrane association of p21 Ras. In 1998, Lee et al[15] reported for the first time that garlic extract, MAT, displayed an inhibitory activity against GGTase I. They determined the IC_{50} of MAT to be 53 μmol/L,[15] the concentration used in the present study.

Garlic has also been purported to play an anti-atherogenic role.[28-34] Extracts of garlic have been shown to reduce serum cholesterol levels, inhibit cholesterol biosynthesis, suppress low-density lipoprotein oxidation, lower plasma fibrinogen, and increase fibrinolytic activity. Campbell et al[29] have demonstrated that aged garlic extract reduced fatty streaks development and aortic arch cholesterol content in rabbits fed a cholesterol-enriched diet without any effect on plasma cholesterol levels. Furthermore, in vitro studies demonstrated that garlic extract completely inhibited proliferation of VSMC.[29] We decided to evaluate its inhibitory activity against geranylgeranylation in the paradigm where PDGF and hyperinsulinemia stimulate VSMC migration. MAT has indeed displayed a strong anti-GGTase I action (Figs 3 and 4) and completely blocked VSMC migration (Figs 1 and 2), suggesting that the potential anti-atherogenic effects of garlic may be related to its anti–GGTase I activity. In contrast to its anti–GGTase I activity, MAT had no effect on either insulin or PDGF signaling to MAPK and Akt (Fig 5). Thus, an anti-atherogenic effectiveness of garlic and its active ingredient, thiosulfinates, is most likely to be related to its inhibitory action on prenylation of Rho proteins. Whether or not orally administered garlic or its extract would display a similarly measurable and significant anti–GGTase I activity remains to be determined.

In summary, these experiments demonstrate that the garlic extract, MAT, completely blocks the potentiating influence of hyperinsulinemia on the PDGF-induced migration of VSMC, thus providing insight into the potential anti-atherogenic influence of garlic.

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