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Intracellular signal transduction modulating expression of plasminogen activator inhibitor-1 in adipocytes

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Abstract

The concentrations in blood of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of fibrinolysis and proteolysis, are elevated in obese and insulin-resistant subjects, predispose them to the risk of thrombosis, and may accelerate atherogenesis. Adipose tissue is a prominent source. Accordingly, intracellular signaling pathways that may influence PAI-1 expression in adipocytes have been the focus of considerable study. Rho, a small GTP binding and GTPase protein, when activated in turn activates its target, Rho-associated coiled-coil forming protein, to yield an active kinase, Rho-kinase, an effector in the Rho pathway. Rho-kinase exerts calcium-sensitizing effects in vascular smooth muscle cells and inhibitory effects on transforming growth factor-β (TGF-β) expression in chicken embryonic heart cells. Because TGF-β is a powerful agonist of PAI-1 expression, we characterized the effects of inhibition of Rho-kinase in 3T3-L1 adipocytes. PAI-1 mRNA was determined by Northern blotting, and PAI-1 protein was determined by Western blotting. The Rho-kinase inhibitor, Y-27632 [(*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide], increased PAI-1 expression markedly. Although genistein, a flavonoid tyrosine kinase, attenuated the increase of PAI-1 induced by Y-27632, other non-flavonoid tyrosine kinase inhibitors did not. However, another flavonoid, daidzein, which lacks tyrosine kinase activity, decreased basal PAI-1 expression and attenuated the induction of PAI-1 expression by Y-27632. Thus, the Rho/Rho-kinase system inhibits PAI-1 expression by a flavonoid-sensitive mechanism in adipocytes. Therefore, flavonoids may be useful in decreasing elevated PAI-1 expression in adipose tissue and its consequent pathophysiologic sequelae.

Keywords: Adipocyte; Rho-kinase; Fibrinolysis; Thrombosis; Obesity; Flavonoid

1. Introduction

PAI-1 plays a pivotal role in fibrinolysis in blood and proteolysis in tissues. It inactivates both tissue-type (t-PA) and urokinase-type (u-PA) plasminogen activators irreversibly [1–3]. An elevated concentration of PAI-1 in blood is a marker and perhaps a major determinant of cardiovascular risk and presages recurrent myocardial infarction and postoperative deep vein thrombosis [4,5].

Elevated concentrations of PAI-1 are seen with insulin-resistant states including type 2 diabetes and obesity, and are associated with an increased risk of thromboembolic events. Adipocytes secrete PAI-1 and appear to contribute to the elevated PAI-1 in blood seen under these conditions [6–10].

We have shown previously that adipocytes stimulated with TGF-β produce PAI-1 [11]. Small GTP-binding and GTPase proteins, such as Rho family members, have been shown to down-regulate TGF-β expression in chicken embryonic heart cells. This pathway is involved in diverse processes in a myriad of cell types [12] including cytoskeletal reorganization [13,14] and transcriptional activation [15]. Its potential influence on PAI-1 expression is controversial [16]. Rho-mediated responses depend on the availability of geranylgeranylated Rho and its activation by GTP loading. In cultured heart cells, HMGCoA reductase inhibitors up-regulate TGF-β signaling and PAI-1 expression [17]. Small G protein Rho-associated coiled-coil

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Abbreviations: HMGCoA, 3-hydroxy-3-methylglutaryl CoA; MAP, mitogen-activated protein; PAI-1, plasminogen activator inhibitor-1; TBS, Tris-buffered saline; TGF-β, transforming growth factor-β.

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forming protein kinase (Rho-kinase), an effector of activated Rho [18,19], inhibits myosin light chain phosphatase [20,21], and alters the actin cytoskeleton [22]. Because of its effects on TGF-β signaling in embryonic chicken heart cells [17], we studied the effects of inhibition of Rho-kinase on the expression of PAI-1 in adipocytes and characterized the potential molecular mechanisms involved.

2. Materials and methods

2.1. Materials

3T3-L1 mouse preadipocytes were obtained from Dainippon, and Dulbecco's modified Eagle's medium (DMEM), isobutylxanthine, and tyrphostin 23 from Sigma. Calf serum was purchased from HyClone; sheep anti-mouse PAI-1 IgG from American Diagnostica; GF109203X (2-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide] from Tocris; and herbimycin A from Alexis. Genistein and daidzein were obtained from Nakarai; PD 98059 (2′-amino-3′-methoxyflavone) and SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1*H*-imidazole] from Calbiochem; and Y-27632 27632 [(*R*)-(+)-*trans*-*N*-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide], an inhibitor of Rho-kinase, from Welfide.

2.2. Cell culture

3T3-L1 preadipocytes were cultured in high glucose (4500 mg/L) DMEM supplemented with 10% serum, 50 U/mL of penicillin and 50 μg/mL of streptomycin until confluent. The medium was changed every 2 days. 3T3-L1 preadipocytes were converted from postconfluent 3T3-L1 preadipocytes to adipocytes as previously described [23]. In brief, the 3T3-L1 preadipocytes were stimulated with 0.25 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 0.9 μM insulin, and 20% serum for 48 hr. The cells were then cultured with DMEM containing 0.9 μM insulin and 20% fetal bovine serum for 5 days. They were differentiated by day 7 and exhibited intracellular lipid

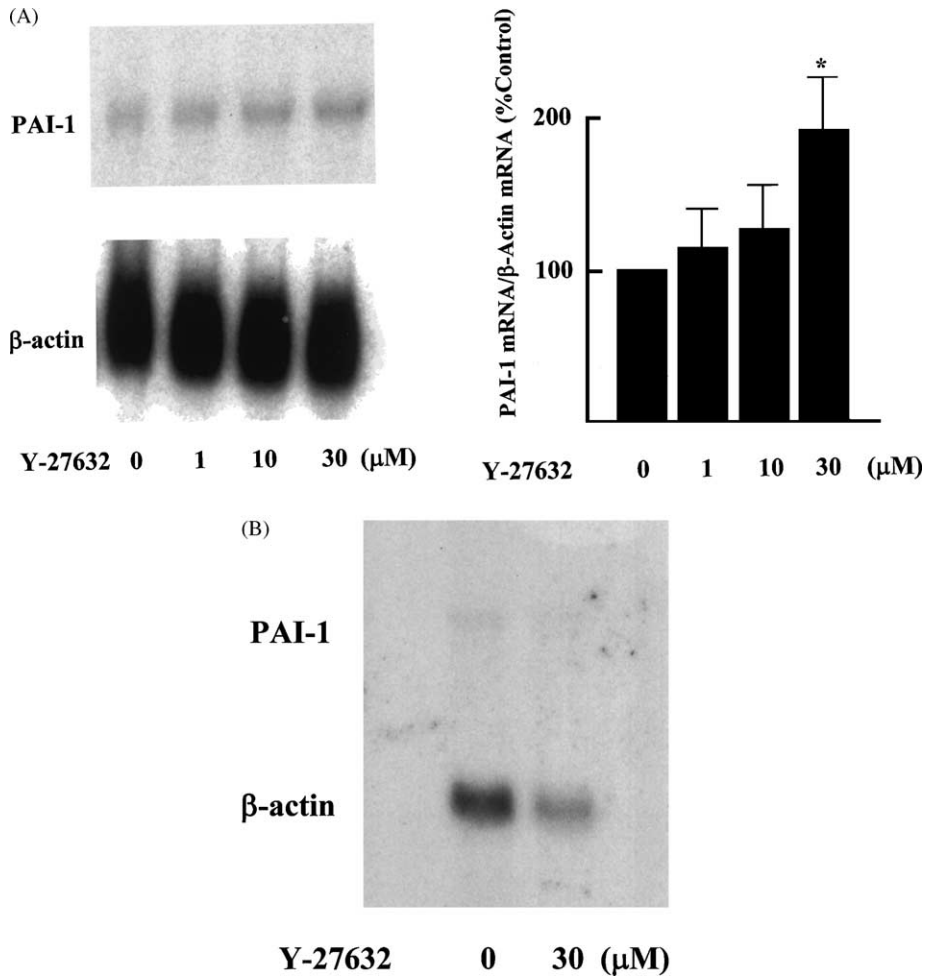


Fig. 1. Effect of Y-27632, a specific inhibitor of Rho-kinase, on PAI-1 mRNA expression. (A, right panel) Well-differentiated 3T3-L1 adipocytes were treated with Y-27632 (0–30 μM) for 12 hr. PAI-1 mRNA was detected by Northern blotting. Data demonstrate percent stimulation (means ± SD) in three independently performed experiments. Key: (*) $P < 0.05$ compared with control. (A, left panel) A representative Northern blot is shown. (B) Preadipocytes were treated with Y-27632 (0 or 30 μM) for 12 hr. A representative Northern blot from three separate experiments is shown.

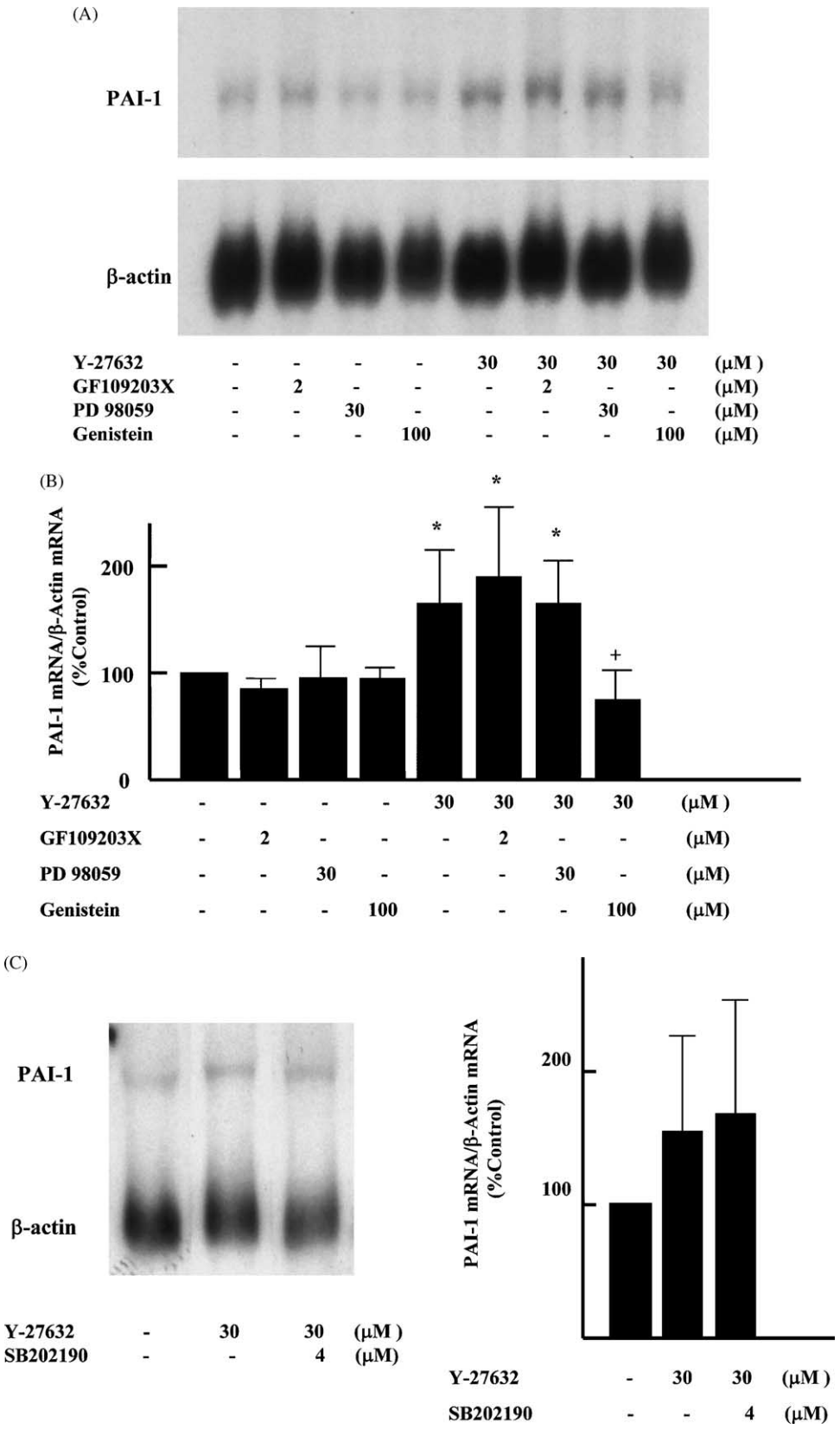
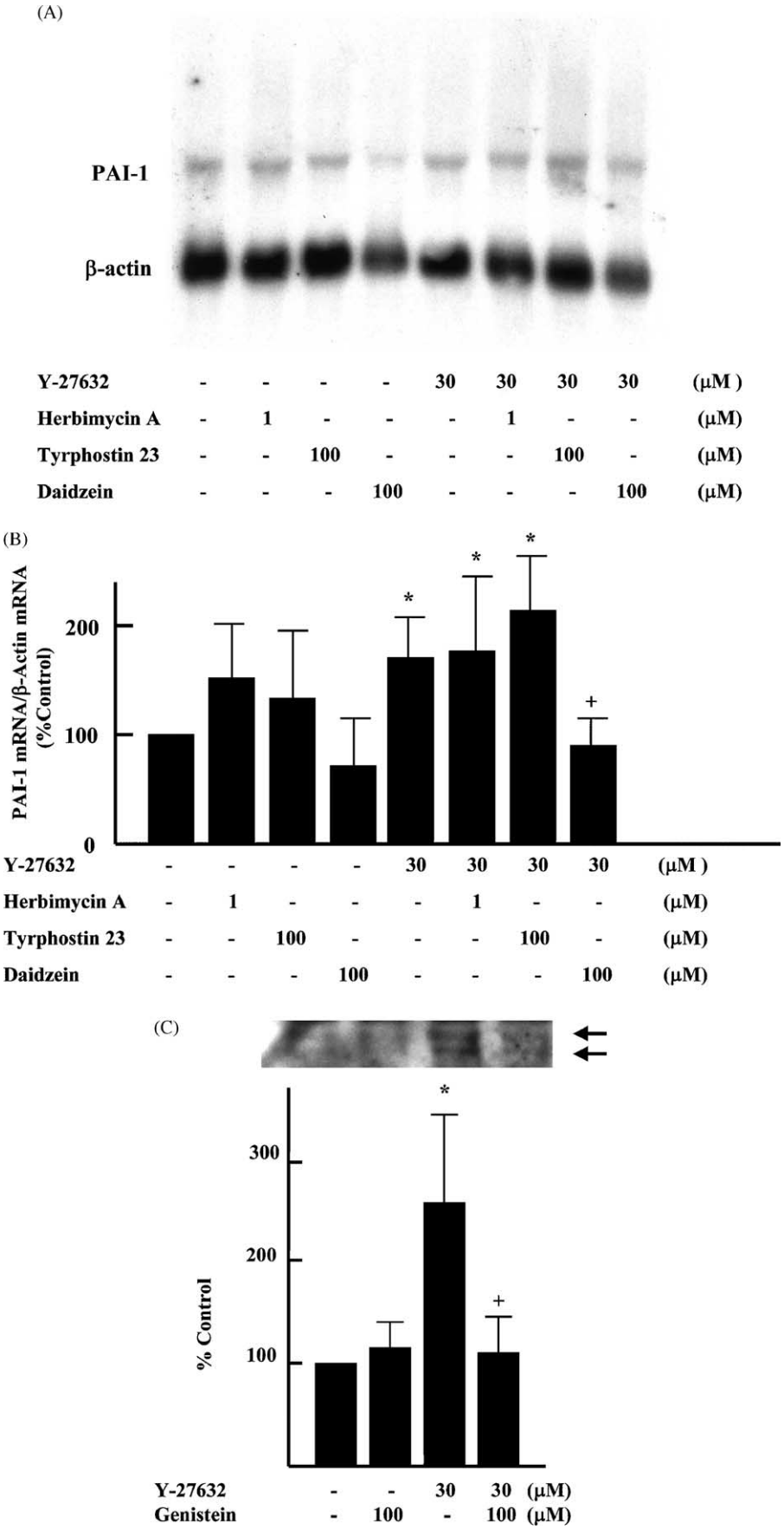


Fig. 2. Effects of genistein, GF109203X, PD98059, and SB202190 on PAI-1 mRNA expression induced by Y-27632 in adipocytes. (A) A representative Northern blot showing the effect of genistein (100 μM), GF109203X (2 μM) and PD98059 (30 μM) on PAI-1 mRNA expression induced by Y-27632 (30 μM). (B) A bar graph showing the effect of genistein (100 μM), GF109203X (2 μM) and PD98059 (30 μM) on PAI-1 mRNA expression induced by Y-27632 (30 μM). Data demonstrate percent stimulation (means ± SD) in three independently performed experiments. Key: (*) $P < 0.05$ compared with control; and (+) $P < 0.05$ compared with Y-27632 (30 μM). (C) A bar graph showing the effect of SB202190 (4 μM) on PAI-1 mRNA expression induced by Y-27632 (30 μM). A representative Northernern blot from three independently performed experiments is shown in the left panel.


(A)
PAI-1
β-actin
Y-27632 - - - - 30 30 30 30 (μM)
Herbimycin A - 1 - - - 1 - - (μM)
Tyrphostin 23 - - 100 - - - 100 - (μM)
Daidzein - - - 100 - - - 100 (μM)
(B)
PAI-1 mRNA/β-Actin mRNA (%Control)
200
100
0
*
*
*
+
Y-27632 - - - - 30 30 30 30 (μM)
Herbimycin A - 1 - - - 1 - - (μM)
Tyrphostin 23 - - 100 - - - 100 - (μM)
Daidzein - - - 100 - - - 100 (μM)
(C)
% Control
300
200
100
*
+
Y-27632 - - 30 30 (μM)
Genistein - 100 - 100 (μM)

droplets. They were used after an additional 1-week incubation.

2.3. Isolation of RNA and Northern blot analysis

Well-differentiated 3T3-L1 adipocytes were preincubated for 24 hr in DMEM without serum followed by stimulation with the Rho-kinase inhibitor, Y-27632, in the presence or absence of GF109203X, PD98059, SB202190, and genistein to identify signal transduction components that may affect PAI-1 expression. RNA was isolated with the use of the acid guanidinium thiocyanate-phenol-chloroform method and separated by 1% agarose gel electrophoresis. RNA was then transferred from the gels to Hybond N^+ nylon membranes (Amersham) and immobilized by UV cross-linking. Blots were hybridized with PAI-1 with the use of a radiolabeled specific cDNA probe as previously described [11]. A housekeeping gene probe, β-actin, was used as an internal control. Autoradiography was performed, and band intensities were quantified by densitometric analysis.

2.4. Western blotting

Concentrations of PAI-1 in conditioned medium were determined with the use of Western blotting with antibody specific for the antigen as described previously with modest modifications [24]. Briefly, conditioned medium was diluted 1:1 with reduced sample buffer (0.125 M Tris–HCl, pH 6.8, 10% sucrose, 4% SDS, 10% 2-mercaptoethanol, and 0.004% bromphenol blue), heated at 100° for 3 min, cooled, and loaded on 8% polyacrylamide gels. Proteins were electrophoresed for 60 min and transferred to polyvinylidene difluoride membranes that were then blocked with 1% BSA and 0.1% Tween 20 in TBS (pH 7.4). Membranes were washed with 0.5% BSA and 0.1% Tween 20 in TBS several times and incubated with 1% BSA and 0.1% Tween 20 in TBS with 2 μg/mL of sheep anti-mouse PAI-1 IgG. Membranes were washed with 0.5% BSA and 0.1% Tween 20 in TBS several times and incubated with alkaline phosphatase-conjugated goat anti-sheep IgG diluted 1:5000 with 1% BSA and 0.1% Tween 20 in TBS. Membranes were incubated with chemiluminescent substrate and exposed to X-ray film. Bands on the developed film were quantified with the use of a densitometer.

2.5. Statistical analysis

Results are expressed as means ± SD. Differences between groups were tested with Student's *t*-test for unpaired data. A P value of <0.05 was regarded as significant.

3. Results

Well-differentiated 3T3-L1 adipocytes were used to characterize the effects of Y-27632, a specific inhibitor of Rho-kinase, on PAI-1 expression. The cells were exposed to Y-27632 (1–30 μM) for 12 hr. PAI-1 mRNA was detectable in untreated cells. Inhibition of Rho-kinase by Y-27632 increased PAI-1 mRNA in a concentration-dependent manner (113 ± 28% over control at 1 μM, 124 ± 29% at 10 μM, and 190 ± 35% at 30 μM, respectively) (Fig. 1A). By contrast, the PAI-1 signal at baseline was faint in preadipocytes, and Y-27632 (30 μM) did not affect PAI-1 mRNA expression in these cells (Fig. 1B).

The mechanism responsible for the increase in PAI-1 expression induced by Y-27632 was evaluated. Pretreatment of adipocytes with a tyrosine kinase inhibitor (genistein, 100 μM) attenuated the increase of PAI-1 mRNA expression induced by 30 μM Y-27632 (72 ± 26% of control) (Fig. 2A and B). By contrast, the protein kinase C inhibitor GF109203X (2 μM) and the mitogen-activated protein (MAP) kinase kinase inhibitor PD98059 (30 μM) did not attenuate the increased PAI-1 expression induced by inhibition of Rho-kinase by Y-27632 (30 μM). These inhibitors had no effect on basal PAI-1 expression. Thus, genistein appeared to inhibit the signaling pathway unmasked by the effects of inhibition of Rho-kinase. The p38 MAP kinase inhibitor SB202190 (4 μM) exerted no effect on the increased PAI-1 mRNA expression induced by the inhibition of Rho-kinase with Y-27632 (30 μM) (165 ± 87% of control, Fig. 2C).

Because genistein is not only a tyrosine kinase inhibitor but also a flavonoid [25], the effects of other tyrosine kinase inhibitors and of a flavonoid were investigated. Use of two other tyrosine kinase inhibitors, herbimycin A (1 μM) and tyrphostin 23 (100 μM), both non-flavonoid tyrosine kinase inhibitors, did not attenuate PAI-1 induction induced by Y-27632 (30 μM) (Fig. 3A and B), suggesting that genistein exerted its inhibitory effect through a

Fig. 3. Effect of herbimycin A, tyrphostin 23, daidzein, and genistein on PAI-1 expression induced by Y-27632 in adipocytes. (A) A representative Northern blot showing the effects of the non-flavonoid tyrosine kinase inhibitors herbimycin A (1 μM) and tyrphostin 23 (100 μM), and the flavonoid daidzein (100 μM), on PAI-1 mRNA expression induced by Y-27632 (30 μM). (B) A bar graph showing the effects of the non-flavonoid tyrosine kinase inhibitors herbimycin A (1 μM) and tyrphostin 23 (100 μM), and the flavonoid daidzein (100 μM), on PAI-1 mRNA expression induced by Y-27632 (30 μM). The data demonstrate percent stimulation (means ± SD) in four independently performed experiments. Key: (*) $P < 0.05$ compared with control; and (+) $P < 0.05$ compared with Y-27632 (30 μM). (C) The effects of Y-27632 (30 μM) and genistein (100 μM) on the accumulation of PAI-1 protein in adipocyte conditioned medium. The data demonstrate percent stimulation (means ± SD) in three independently performed experiments. Key: (*) $P < 0.05$ compared with control; and (+) $P < 0.05$ compared with Y-27632 (30 μM). A representative Western blot is shown in the upper panel.

non-tyrosine kinase mechanism. In keeping with this possibility, daidzein (100 μM), another flavonoid with only weak tyrosine kinase inhibition, decreased basal PAI-1 expression and attenuated PAI-1 expression induced by Y-27632 (30 μM) ($66 \pm 43\%$ and $88 \pm 25\%$ compared with control, respectively). Accumulation of PAI-1 protein in adipocyte conditioned medium was increased by Y-27632 (30 μM) as assessed by Western blotting ($258 \pm 86\%$ over control at 30 μM) (Fig. 3C). Genistein (100 μM) completely inhibited the increase of PAI-1 induced by Y-27632 ($109 \pm 37\%$ of control).

4. Discussion

Adipose tissue is a probable source of PAI-1 in the blood of patients with insulin-resistant states including type 2 diabetes and obesity. In this study, PAI-1 expression was increased in adipocytes compared with that in preadipocytes. The results are compatible with the concept that adipocytes are one of the major cell types responsible for PAI-1 secretion into blood. Inhibition of Rho-kinase by Y-27632 increased PAI-1 mRNA expression in a concentration-dependent manner. In human fat tissue, preadipocytes and stromal cells may also contribute to PAI-1 production [26].

TGF-β is a potent inducer of PAI-1 in human adipocytes [27] and cultured heart cells [17]. HMGCoA reductase inhibitors up-regulate both TGF-β signaling and PAI-1 expression through inhibition of the geranylgeranylation of a Rho-family member, RhoA GTPase [17]. Thus, it is likely that Y-27632 could increase PAI-1 expression by up-regulating TGF-β signaling in adipocytes, or more specifically by de-repressing Rho pathway-dependent suppression of TGF-β expression although adipocyte TGF-β expression was not determined in this study. Because angiotensin II-induced *PAI-1* gene expression is inhibited by Y-27632 in heart *in vivo* [28] and in cultured smooth muscle cells *in vitro* [29] and because Rho-kinase inhibitors suppress PAI-1 synthesis in monocytes [30], the effects of Y-27632 may be cell- or organ-specific and dependent upon the agonists used.

The tyrosine kinases, protein kinase C and MAP kinase may influence PAI-1 expression in mesangial cells and $HepG_2$ cells [31,32]. In adipocytes, pretreatment with genistein attenuated the increase of PAI-1 mRNA expression induced by Y-27632. By contrast, the protein kinase C inhibitor GF109203X, the MAP kinase kinase inhibitor PD98059, and the p38 MAP kinase inhibitor SB202190 exerted no effects on the increased PAI-1 mRNA expression induced by inhibition of Rho-kinase. Therefore, it is likely that a genistein-sensitive mechanism that we identified is involved in the cell signaling after inhibition of Rho-kinase with Y-27632. Inhibitors were used at the concentrations previously used in adipocytes [33–36]. However, higher concentrations may result in different responses [36].

Genistein reduces the induction of PAI-1 by tumor necrosis factor-α, basic fibroblast growth factor, insulin, and IGF-1 [37–39]. Yet other tyrosine kinase inhibitors (herbimycin A and tyrphostin 23) did not reduce induction of PAI-1 in the present study. These results are consistent with the likelihood that genistein was not acting as a tyrosine kinase inhibitor but rather exerted its effects via a different mechanism. Genistein is not only a tyrosine kinase inhibitor but also a flavonoid. It exerts diverse effects in adipocytes such as inhibition of mitotic clonal expansion, triglyceride accumulation and peroxisome proliferator-activated receptor-γ [25]. Because we found that another flavonoid also decreases PAI-1 expression, it appears that flavonoids themselves can inhibit PAI-1 expression in adipocytes. Flavonoids are polyphenolic compounds that exist widely in plants, inhibit the proliferation of tumor and non-tumor cells in culture, induce apoptosis, exert estrogenic and antiestrogenic effects, and function as antioxidants [40]. Flavonoids alter the activity of a number of intracellular enzymes, including tyrosine kinases [41]. Primarily because of their antiproliferative effects, flavonoids have been a focus of active exploration seeking to identify anti-cancer agents. Genistein, a soy isoflavone, inhibits the proliferation of a number of cancer cell lines and commonly induces differentiation. In this regard, it is of interest that flavonoids can function as antioxidants and that oxidative stress can induce PAI-1 in adipocytes as we previously reported [24]. It is not clear at the mechanistic level how the flavonoids regulate the Rho/Rho-kinase system. It is of interest that activation of a Rho-like small G protein is related to oxygen deprivation tolerance in plants [42].

Results in this study suggest that flavonoids may provide a means for down-regulating PAI-1 expression, thereby diminishing cardiovascular risk in conditions such as obesity and insulin-resistant states in general. The effect they exert on the expression of PAI-1 in adipocytes appears to reflect an impact on the Rho-kinase pathway, perhaps through activating the pathway and thereby suppressing expression of TGF-β and its induction of PAI-1. Modulation of activity of the Rho/Rho-kinase pathway and its effect on *PAI-1* gene expression by a genistein-sensitive mechanism in adipocytes suggests that pharmacological interventions with flavonoids may be beneficial in decreasing PAI-1 expression and reducing thrombotic events in patients with diverse insulin-resistant states including type 2 diabetes mellitus and obesity.

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References

[1] Lucore CL, Sobel BE. Interactions of tissue-type plasminogen activator with plasma inhibitors and their pharmacologic implications. Circulation 1988;77:660–9.

[2] Wagner OF, de Vries C, Hohmann C, Veerman H, Pannekoek H. Interaction between plasminogen activator inhibitor type 1 (PAI-1) bound to fibrin and either tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). Binding of t-PA/PAI-1 complexes to fibrin mediated by both the finger and the kringle-2 domain of t-PA. J Clin Invest 1989;84:647–55.

[3] Sakata Y, Okada M, Noro A, Matsuda M. Interaction of tissue-type plasminogen activator and plasminogen activator inhibitor 1 on the surface of endothelial cells. J Biol Chem 1988;263:1960–9.

[4] Wiman B. Plasminogen activator inhibitor 1 (PAI-1) in plasma: its role in thrombotic disease. Thromb Haemost 1995;74:71–6.

[5] Paramo JA, Alfaro MJ, Rocha E. Postoperative changes in the plasmatic levels of tissue-type plasminogen activator and its fast-acting inhibitor—relationship to deep vein thrombosis and influence of prophylaxis. Thromb Haemost 1985;54:713–6.

[6] Loskutoff DJ, Samad F. The adipocyte and hemostatic balance in obesity: studies of PAI-1. Arterioscler Thromb Vasc Biol 1998;18:1–6.

[7] Mavri A, Stegnar M, Krebs M, Sentocnik JT, Geiger M, Binder BR. Impact of adipose tissue on plasma plasminogen activator inhibitor-1 in dieting obese women. Arterioscler Thromb Vasc Biol 1999;19:1582–7.

[8] Morange PE, Alessi MC, Verdier M, Casanova D, Magalon G, Juhan-Vague I. PAI-1 produced ex vivo by human adipose tissue is relevant to PAI-1 blood level. Arterioscler Thromb Vasc Biol 1999;19:1361–5.

[9] Crandall DL, Busler DE, McHendry-Rinde B, Groeling TM, Kral JG. Autocrine regulation of human preadipocyte migration by plasminogen activator inhibitor-1. J Clin Endocrinol Metab 2000;85:2609–14.

[10] Samad F, Yamamoto K, Loskutoff DJ. Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue *in vivo*. Induction by tumor necrosis factor-α and lipopolysaccharide. J Clin Invest 1996;97:37–46.

[11] Brown SL, Sobel BE, Fujii S. Attenuation of the synthesis of plasminogen activator inhibitor type 1 by niacin. A potential link between lipid lowering and fibrinolysis. Circulation 1995;92:767–72.

[12] Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. Physiol Rev 2001;81:153–208.

[13] Miura Y, Kikuchi A, Musha T, Kuroda S, Yaku H, Sasaki T, Takai Y. Regulation of morphology by *rho* p21 and its inhibitory GDP/GTP exchange protein (*rho* GDI) in Swiss 3T3 cells. J Biol Chem 1993;268:510–5.

[14] Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992;70:389–99.

[15] Hill CS, Wynne J, Treisman R. The Rho family GTPases RhoA, Rac1, and CDC42Hs regulate transcriptional activation by SRF. Cell 1995;81:1159–70.

[16] Essig M, Nguyen G, Prie D, Escoubet B, Sraer JD, Friedlander G. 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors increase fibrinolytic activity in rat aortic endothelial cells. Role of geranylgeranylation and Rho proteins. Circ Res 1998;83:683–90.

[17] Park HJ, Galper JB. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors up-regulate transforming growth factor-β signaling in cultured heart cells via inhibition of geranylgeranylation of RhoA GTPase. Proc Natl Acad Sci USA 1999;96:11525–30.

[18] Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO J 1996;15:1885–93.

[19] Leung T, Manser E, Tan L, Lim L. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem 1995;270:29051–4.

[20] Hirose M, Ishizaki T, Watanabe N, Uehata M, Kranenburg O, Moolenaar WH, Matsumura F, Maekawa M, Bito H, Narumiya S. Molecular dissection of the Rho-associated protein kinase (p160ROCK)-regulated neurite remodeling in neuroblastoma N1E-115 cells. J Cell Biol 1998;141:1625–36.

[21] Fukiage C, Mizutani K, Kawamoto Y, Azuma M, Shearer TR. Involvement of phosphorylation of myosin phosphatase by ROCK in trabecular meshwork and ciliary muscle contraction. Biochem Biophys Res Commun 2001;288:296–300.

[22] Maekawa M, Ishizaki T, Boku S, Watanabe N, Fujita A, Iwamatsu A, Obinata T, Ohashi K, Mizuno K, Narumiya S. Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. Science 1999;285:895–8.

[23] Guo X, Liao K. Analysis of gene expression profile during 3T3-L1 preadipocyte differentiation. Gene 2000;251:45–53.

[24] Sakamoto T, Woodcock-Mitchell J, Marutsuka K, Mitchell JJ, Sobel BE, Fujii S. TNF-α and insulin, alone and synergistically, induce plasminogen activator inhibitor-1 expression in adipocytes. Am J Physiol 1999;276:C1391–7.

[25] Harmon AW, Harp JB. Differential effects of flavonoids on 3T3-L1 adipogenesis and lipolysis. Am J Physiol 2001;280:C807–13.

[26] Bastelica D, Morange P, Berthet B, Borghi H, Lacroix O, Grino M, Juhan-Vague I, Alessi MC. Stromal cells are the main plasminogen activator inhibitor-1-producing cells in human fat: evidence of differences between visceral and subcutaneous deposits. Arterioscler Thromb Vasc Biol 2002;22:173–8.

[27] Birgel M, Gottschling-Zeller H, Rohrig K, Hauner H. Role of cytokines in the regulation of plasminogen activator inhibitor-1 expression and secretion in newly differentiated subcutaneous human adipocytes. Arterioscler Thromb Vasc Biol 2000;20:1682–7.

[28] Kobayashi N, Nakano S, Mita S, Kobayashi T, Honda T, Tsubokou Y, Matsuoka H. Involvement of Rho-kinase pathway for angiotensin II-induced plasminogen activator inhibitor-1 gene expression and cardiovascular remodeling in hypertensive rats. J Pharmacol Exp Ther 2002;301:459–66.

[29] Takeda K, Ichiki T, Tokunou T, Iino N, Fujii S, Kitabatake A, Shimokawa H, Takeshita A. Critical role of Rho-kinase and MEK/ERK pathways for angiotensin II-induced plasminogen activator inhibitor type-1 gene expression. Arterioscler Thromb Vasc Biol 2001;21:868–73.

[30] Ishibashi T, Nagata K, Ohkawara H, Sakamoto T, Yokoyama K, Shindo J, Sugimoto K, Sakurada S, Takuwa Y, Teramoto T, Maruyama Y. Inhibition of Rho/Rho-kinase signaling down-regulates plasminogen activator inhibitor-1 synthesis in cultured human monocytes. Biochim Biophys Acta 2002;1590:123–30.

[31] Motojima M, Kakuchi J, Yoshioka T. Association of TGF-β signaling in angiotensin II-induced PAI-1 mRNA up-regulation in mesangial cells: role of PKC. Biochim Biophys Acta 1999;1449:217–26.

[32] Arts J, Grimbergen J, Toet K, Kooistra T. On the role of c-Jun in the induction of PAI-1 gene expression by phorbol ester, serum, and IL-1α in HepG2 cells. Arterioscler Thromb Vasc Biol 1999;19:39–46.

[33] Wu-Wong JR, Berg CE, Wang J, Chiou WJ, Fissel B. Endothelin stimulates glucose uptake and GLUT4 translocation via activation of endothelin ETA receptor in 3T3-L1 adipocytes. J Biol Chem 1999;274:8103–10.

[34] Kitamura T, Kimura K, Jung BD, Makondo K, Okamoto S, Canas X, Sakane N, Yoshida T, Saito M. Proinsulin C-peptide rapidly stimulates mitogen-activated protein kinases in Swiss 3T3 fibroblasts: requirement of protein kinase C, phosphoinositide 3-kinase and pertussis toxin-sensitive G-protein. Biochem J 2001;355:123–9.

[35] Konrad D, Somwar R, Sweeney G, Yaworsky K, Hayashi M, Ramlal T, Klip A. The antihyperglycemic drug α-lipoic acid stimulates glucose uptake via both GLUT4 translocation and GLUT4 activation: potential role of p38 mitogen-activated protein kinase in GLUT4 activation. Diabetes 2001;50:1464–71.

[36] Kandulska K, Nogowski L, Szkudelski T. Effect of some phytoestrogens on metabolism of rat adipocytes. Reprod Nutr Dev 1999;39:497–501.

[37] Schneider DJ, Nordt TK, Sobel BE. Stimulation by proinsulin of expression of plasminogen activator inhibitor type-I in endothelial cells. Diabetes 1992;41:890–5.

[38] Fotsis T, Pepper M, Adlercreutz H, Fleischmann G, Hase T, Montesano R, Schweigerer L. Genistein, a dietary-derived inhibitor of *in vitro* angiogenesis. Proc Natl Acad Sci USA 1993;90:2690–4.

[39] van Hinsbergh VWM, Vermeer M, Koolwijk P, Grimbergen J, Kooistra T. Genistein reduces tumor necrosis factor α-induced plasminogen activator inhibitor-1 transcription but not urokinase expression in human endothelial cells. Blood 1994;84:2984–29891.

[40] Tikkanen MJ, Adlercreutz H. Dietary soy-derived isoflavone phytoestrogens. Could they have a role in coronary heart disease prevention? Biochem Pharmacol 2000;60:1–5.

[41] Hagiwara M, Inoue S, Tanaka T, Nunoki K, Ito M, Hidaka H. Differential effects of flavonoids as inhibitors of tyrosine protein kinases and serine/threonine protein kinases. Biochem Pharmacol 1988;37:2987–92.

[42] Baxter-Burrell A, Yang Z, Springer PS, Bailey-Serres J. RopGAP4-dependent Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. Science 2002;296:2026–8.