

Riccardin C: A natural product that functions as a liver X receptor (LXR) α agonist and an LXR β antagonist

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Abstract Liver X receptors (LXRs) α and β share considerable sequence homology and several functions, respond to the same endogenous and synthetic ligands, and play critical roles in maintaining lipid homeostasis. In this study, liverwort-derived riccardin C (RC) and F (RF) were identified as an LXR α agonist/LXR β antagonist and an LXR α antagonist, respectively. RC and RF bound to LXRs, but had different abilities to recruit a coactivator and thereby induce transactivation. Despite its unique subtype-selective activity, RC enhanced ABCA1 and ABCG1 expression and cellular cholesterol efflux in THP-1 cells. RC may provide a novel tool for identifying subtype-function and drug development.

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Keywords: Liver X receptor; ATP-binding cassette transporter A1; ATP-binding cassette transporter G1; HDL; Cholesterol

1. Introduction

The liver X receptors α and β (LXR α and LXR β) are nuclear receptors that form obligate heterodimers with retinoid X receptor (RXR) [1]. LXRs are activated by several oxysterols or intermediates in the cholesterol synthetic pathway [2,3] and serve as key regulators of cholesterol homeostasis by coordinately regulating several genes involved in the efflux, transport, and excretion of cholesterol. These genes include ATP-binding cassette transporter (ABC) A1, ABCG1, and apolipoprotein E – which mediate cellular cholesterol efflux; cholesterol 7 α -hydroxylase – the rate limiting enzyme for the conversion of cholesterol to bile acids in the liver; and ABCG5/ABCG8 – transporters involved in cholesterol/sterol excretion from the liver and intestine [1]. LXR activation also upregulates genes involved in fatty acid and triglyceride synthesis by inducing sterol regulatory element-

binding protein-1c (SREBP-1c) – the master regulator of genes involved in lipogenesis [4–6] – and fatty acid synthase expression [7].

LXR α and LXR β share a high degree of amino acid similarity (78%), have similar binding affinities to physiological oxysterol ligands [2,3], and have several common functions, including the upregulation of ABCA1 and ABCG1 expression [8,9]. However, these receptors have different tissue distributions: while LXR β is ubiquitously expressed, the expression of LXR α is limited to the liver, kidney, intestine, adipose tissue, and macrophages [10]. In the liver, LXR α serves as an important regulator of cholesterol catabolism. Mice lacking LXR α has no resistance to dietary cholesterol and fail to upregulate hepatic cholesterol 7 α -hydroxylase [11,12], whereas LXR β null mice maintain their resistance. The role of LXR subtypes in triglyceride metabolism is uncertain. One study has shown a reduction in the liver triglyceride level of LXR β null mice on standard diet [13], whereas a reduction in the expression of lipogenic genes was observed in LXR α null mice fed cholesterol [11,12]. Thus, selective agonists for each LXR subtype may be necessary to elucidate their precise functions.

Riccardin C (RC) and F (RF) are non-sterol natural products isolated from liverworts [14,15] (Fig. 1A). In the present study, we discovered that RC functions as an LXR α -selective agonist/LXR β antagonist but effectively enhances cholesterol efflux from THP-1 cells.

2. Materials and methods

2.1. Riccardins C and F

RC and RF were purified from a methanol extract of the liverwort *Blasia pusilla* as described previously [14,15].

2.2. Transient transfections and reporter gene assay

CV-1 cells maintained in DMEM containing 10% FCS were co-transfected with 248 ng of LXR response element (LXRE)-driven luciferase vector (pLXRE_X4-tk-Luc), 248 ng of pSV- β -galactosidase control vector (Promega), and 1.25 ng each of pcDNA3.1-LXR α or LXR β and pcDNA3.1-RXR α with Polyfect (Qiagen) in 24-well plates according to a previously described method [16]. Three hours after transfection, the cells were treated with test compounds in DMEM containing 10% delipidated FBS, 20 μ M compactin, and 10 μ M mevalonic acid for 24 h. The cells were then lysed and the reporter gene activity was determined. Luciferase activity was normalized to that of β -galactosidase for each well.

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Abbreviations: LXR, liver X receptor; RXR, retinoid X receptor; ABC, ATP-binding cassette transporter; SREBP-1c, sterol regulatory element-binding protein-1c; LXRE, LXR response element; 22(R)HC, 22(R)-hydroxycholesterol; TO-1317, TO-901317

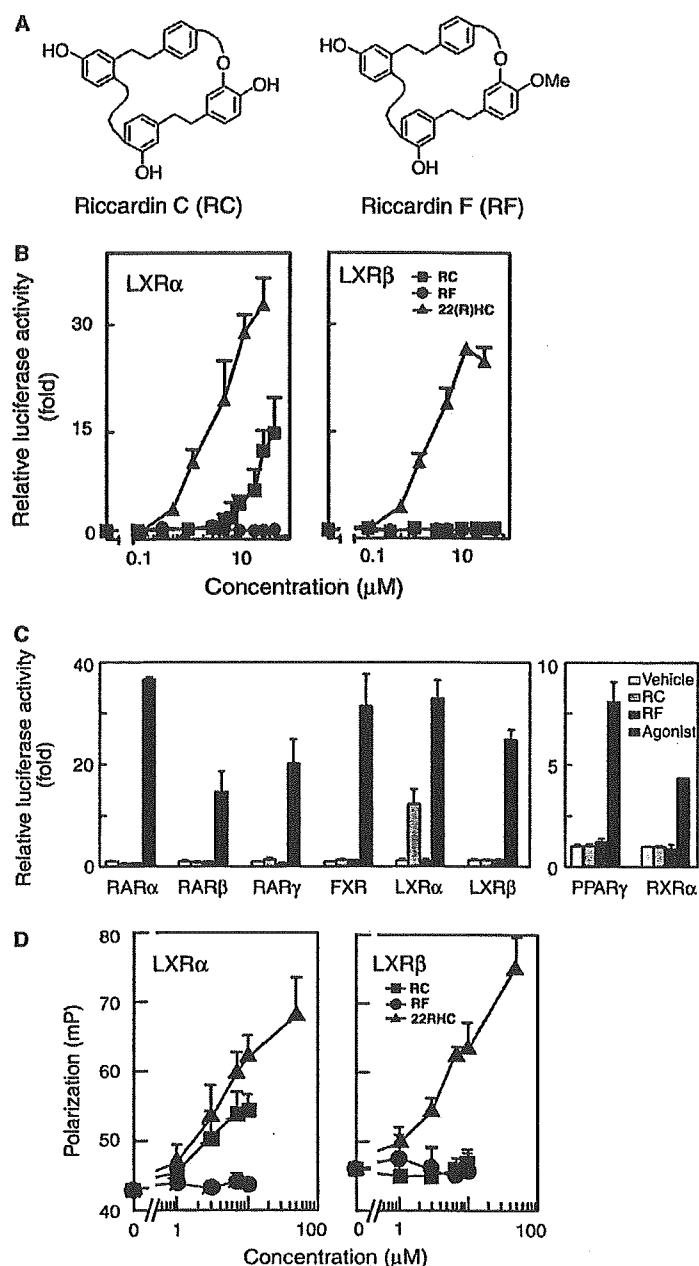


Fig. 1. RC activates LXR α but not LXR β . (A) Chemical structures of RC and RF. (B) CV-1 cells were transfected with a reporter plasmid (pLXRE $_x$ 4-tk-Luc) and expression plasmids for LXR α (or LXR β) and RXR α , together with a β -galactosidase as an internal control, and were treated for 24 h with various concentrations of RC, RF, or 22(R)HC. Luciferase activity in the cell extracts was normalized using β -galactosidase and expressed as the fold induction relative to vehicle-treated cells. The values are the means \pm S.D. of six experiments. (C) Effects of RC and RF on transcriptional activities of heterodimers between RXR α and RAR α , RAR β , RAR γ , FXR, LXR α , or LXR β , and RXR α homodimers. CV-1 cells were transfected with expression plasmids for receptors and a β -galactosidase together with a reporter plasmid (pDR5 $_x$ 3-cmv-Luc for RARs, pFXRE $_x$ 4-tk-Luc, or pDR1 $_x$ 4-cmv-Luc for RXR α), or a PPAR γ -Gal4-expression plasmid with a pGal4-UAS-Luc. The cells were exposed to RC (30 μ M), RF (30 μ M), or receptor-specific agonists (3 μ M all-trans retinoic acid for RARs and RXR α , 30 μ M chenodeoxycholic acid for FXR, 20 μ M 22(R)HC for LXRs, 30 μ M ciglitazone for PPAR γ) for 24 h before assaying luciferase activity. The values are the means \pm S.D. ($n = 3$). (D) Purified GST-LXR α or GST-LXR β ligand binding domain was incubated with a fluorescence-tagged SRC-1 peptide and various concentrations of RC, RF, or 22(R)HC. The association of ligand-induced SRC-1 peptide with the receptor was monitored by evaluating the increases in millipolarization fluorescence units (mP). The values are the means \pm S.D. of six experiments.

2.3. Coactivator association assay using fluorescence polarization

A coactivator association assay using fluorescence polarization was performed according to a previously described method [16]. A TAMRA-labeled peptide (0.1 μ M with the amino acid sequence ILRKLQEQ) was incubated with purified 1.5 μ M GST-fused human LXR α ligand binding domain or LXR β ligand binding domain in

100 μ l of buffer (10 mM HEPES, 150 mM NaCl, 2 mM MgCl $_2$, and 5 mM DTT at pH 7.9) in 96-well black polypropylene plates. After 1-h incubation at room temperature, ligand-dependent recruitment of the coactivator-peptide was measured as the increase in fluorescence polarization using a Fluorescence Plate Reader Fusion α (Perkin-Elmer Life Science).

2.4. Real-time quantitative reverse transcription-PCR

Gene-specific mRNA quantitation was performed by real-time RT-PCR on an ABI Prism 7700 sequence detection system (Applied Biosystems). THP-1 cells maintained in RPMI 1640 medium (Sigma) containing 10% FCS were subcultured in 6-well plates and treated with test compounds in RPMI 1640 containing 0.2% bovine serum albumin. Total RNA extracted from cells using an RNeasy Kit (Qiagen) was treated with DNase according to the manufacturer's instructions. The relative mRNA expression levels were determined using the TaqMan one-step RT-PCR Master Mix (PE Applied Biosystems). The primer/probe sequences for human ABCA1, ABCG1, and SREBP-1c have been previously reported [17].

2.5. Measurement of lipid efflux to apolipoprotein A-I

The lipid efflux measurements were performed according to a previously described method [18]. Briefly, THP-1 cells were treated with the test compounds and 0 or 10 $\mu\text{g}/\text{ml}$ of human apoA-I, isolated from the plasma HDL fraction, in RPMI 1640 containing 0.2% bovine serum albumin for 24 h. The lipids were extracted and the cholesterol level was determined using enzymatic methods.

2.6. Statistical analysis

Data were analyzed by ANOVA followed by the Student–Newman–Keuls method.

3. Results

3.1. Identification of RC as an LXR α Agonist

To discover novel LXR agonists, we screened a variety of natural products using a transient transfection assay. CV-1 cells were cotransfected with an LXRE-driven luciferase reporter plasmid and expression plasmids for human LXR α (or LXR β) and RXR α . RC and RF (Fig. 1A) are novel macrocyclic bis(benzyl) dimers isolated from the liverwort [14,15]. Upon cotransfection with LXR α /RXR, RC (30 μM) raised the transactivation of the reporter gene by approximately 15-fold, while a natural LXR agonist, 22(R)-hydroxycholesterol (22(R)HC), resulted in a 30-fold increase (Fig. 1B). At higher concentrations, RC caused a decrease in β -galactosidase activity. LXR β /RXR α -dependent transactivation was unaffected by this compound. RF, a 14-methoxyl derivative of RC, activated neither LXR α nor LXR β . Furthermore, neither RC nor RF activated heterodimers between RXR α and RAR α , RAR β , RAR γ , FXR, or PPAR γ , and RXR α homodimers in cell-based luciferase assays (Fig. 1C).

In an in vitro coactivator-recruitment assay, RC (up to 10 μM) induced a dose-dependent interaction between SRC-1

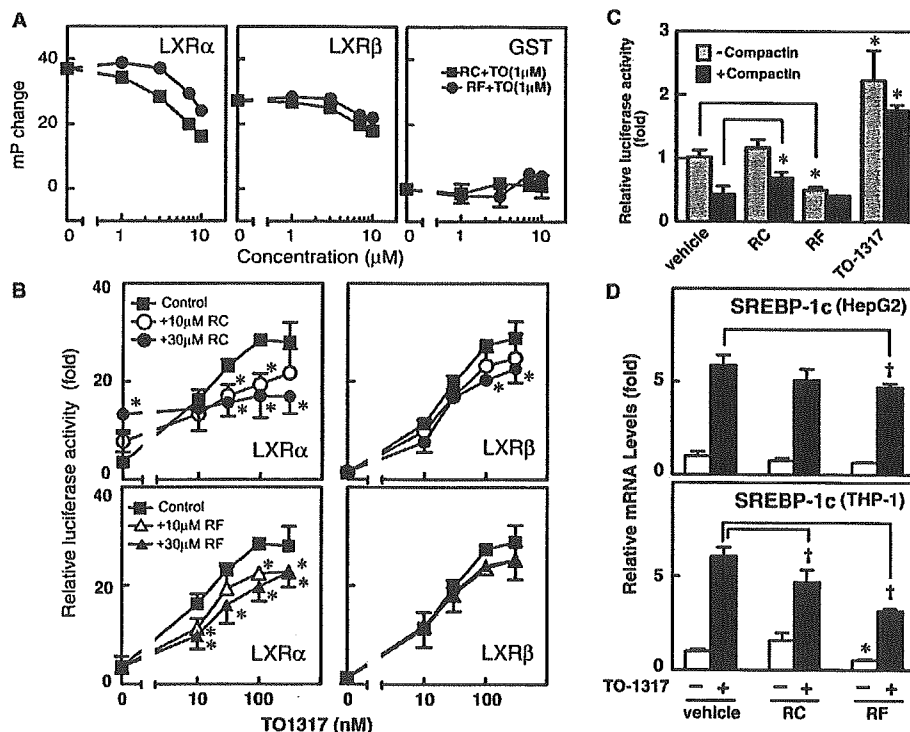


Fig. 2. RC competes with endogenous or synthetic ligands for LXR activation. (A) Fluorescence-tagged SRC-1 peptide was incubated with GST-LXR α , GST-LXR β , or GST. The changes in fluorescence polarization caused by 1 μM TO-1317 in the presence of the indicated concentrations of RC or RF are shown. A blank value in the absence of ligands was subtracted from the measured values. The values are the means \pm S.D. ($n = 3$). (B) CV-1 cells were transfected with pLXRE $_4$ -tk-*Luc* and either LXR α /RXR α or LXR β /RXR α plasmids, as shown in Fig. 1B, and treated for 24 h with various concentrations of TO-1317 with or without RC or RF (10 or 30 μM). The values are the means \pm S.D. of three experiments performed in triplicate. Data were normalized to no ligand control (take as 1) and 100 nM TO-1317 values of a representative experiment. Significantly different from respective controls (*, $P < 0.05$). (C) HepG2 cells were transfected with pLXRE-tk-*Luc* and treated for 24 h with RC (30 μM) or TO-1317 (10 nM) with or without compactin (50 μM) and mevalonic acid (40 μM). The values are the means \pm S.D. of three experiments performed in triplicate. Significantly different from respective controls (*, $P < 0.05$). (D) THP-1 cells and HepG2 cells were treated for 24 h with the vehicle (DMSO) alone, RC or RF (30 μM) in the presence or absence of 100 nM TO-1317. SREBP-1c mRNA level was measured by TaqMan quantitative real-time PCR. Data were normalized to 18S rRNA levels and are expressed as the fold induction relative to that in the vehicle-treated cells. The values are the means \pm S.D. ($n = 3$). Significantly different from vehicle controls (*, $P < 0.05$) or cells treated with TO-1317 alone (\dagger , $P < 0.05$).

peptide and LXR α but not LXR β (Fig. 1D). At higher concentrations, RC caused non-specific interference in monitoring fluorescence polarization. No interactions were induced by RF.

3.2. RC and RF compete with synthetic or endogenous ligands for LXR activation

When assayed in the presence of 1 μ M TO-1317 (TO-901317), RC and RF reduced the TO-1317-induced association of the SRC-1 peptide with LXR α or LXR β , indicating the binding of these compounds to the receptors (Fig. 2A). These compounds also decreased LXR α -transactivation elicited by a synthetic LXR agonist TO-1317 (Fig. 2B), although inhibitory effect of RF on LXR β was insignificant. The ability of RC and RF to compete with endogenous ligands was tested in HepG2 cells. Treatment of the cells with compactin, which has been reported to deplete endogenous ligands for LXR [19,20], led to a 60% reduction in LXRE-dependent transactivation (Fig. 2C). Similarly, RF decreased the luciferase activity to the level of cells treated with compactin. RC had no effect in the absence of compactin but increased luciferase activity by 1.6-fold in compactin-treated cells. RC and RF inhibited the TO-1317-elicited expression of SREBP-1c mRNA in THP-1 cells. RF also decreased the TO-1317-elicited expression in HepG2 cells, although the effect of RC was insignificant (Fig. 2D).

3.3. RC enhances ApoA-I-mediated cellular cholesterol release

We used real-time quantitative PCR to investigate the effect of RC on the expression of LXR target genes, ABCA1, ABCG1, and SREBP-1c, in THP-1 cells. RC (30 μ M) increased the ABCA1 mRNA level by 2-fold, while 22RHC (12.5 μ M) and TO-1317 (10 nM) caused 2.2- and 2.7-fold inductions, respectively (Fig. 3A). Likewise, RC raised the ABCG1 and SREBP-1c mRNA level by 2.6-fold and 1.6-fold, respectively.

To evaluate whether the increase in ABCA1 mRNA expression was functionally relevant, we examined the effect of RC on cholesterol efflux from THP-1 cells. As shown in Fig. 3B, apoA-I-dependent cholesterol release was increased 2-fold by 10 μ M of RC. At 30 μ M, this compound also caused a 2-fold elevation in cholesterol release without exogenous apoA-I.

RC had no effect on SREBP-1c and ABCG1 mRNA expression in HepG2 cells, whereas TO-1317 markedly induced expression of both of them (Fig. 3C).

4. Discussion

In the present study, we identified RC as an LXR α -selective agonist. RC bound directly to LXR α and recruited the coacti-

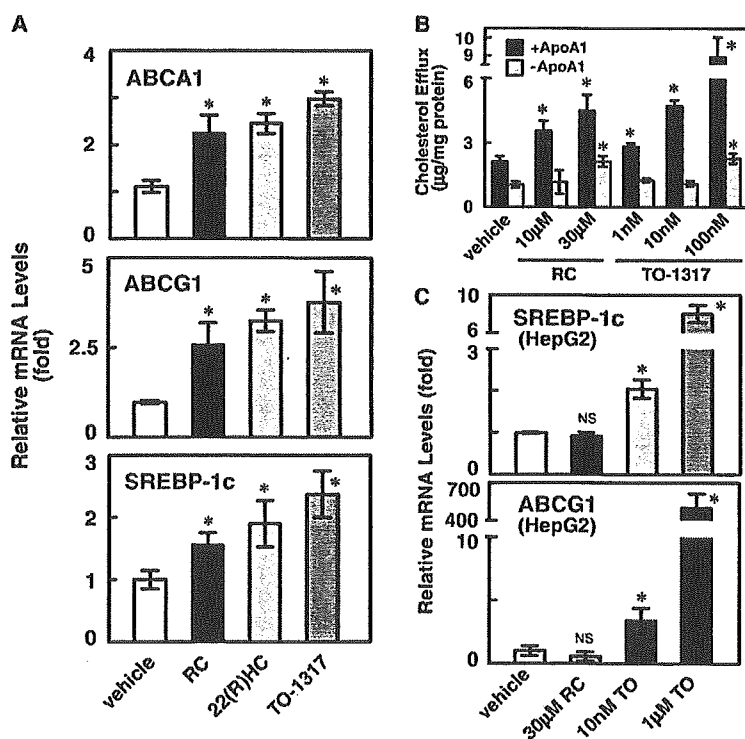


Fig. 3. RC increases ABCA1 and ABCG1 mRNA expression and cellular cholesterol efflux in THP-1 cells without raising SREBP-1c expression in HepG2 cells. (A) THP-1 cells were treated for 24 h with the vehicle (DMSO) alone, RC (30 μ M), TO-1317 (10 nM), or 22(R)HC (12.5 μ M). The levels of ABCA1 and ABCG1 mRNA were measured with TaqMan quantitative real-time PCR analysis. Data were normalized using the 18S rRNA levels and are expressed as the fold induction relative to that in the vehicle-treated cells. The values are the means \pm S.D. of three experiments performed in triplicate. (B) Cells were treated for 24 h with the indicated compounds in the presence or absence of apoA-I (10 μ g/ml), and the cholesterol released into the medium was analyzed. The values are the means \pm S.D. ($n = 3$) of a typical series of three experiments performed. (C) HepG2 cells were treated for 24 h with the vehicle (DMSO) alone, RC (30 μ M), or TO-1317 (10 nM or 1 μ M). SREBP-1c and ABCG1 mRNA level was measured by quantitative real-time PCR. Data were normalized to 18S rRNA levels and are expressed as the fold induction relative to that in the vehicle-treated cells. The values are the means \pm S.D. of three experiments. Statistically significant differences from control are indicated by asterisk (*, $P < 0.05$ and NS, not significant).

vator SRC-1 peptide to the receptor (Fig. 1D), leading to the activation of LXR α -dependent reporter gene transcription (Fig. 1B). RC also possesses the ability to bind to LXR β . However, its inability to recruit a coactivator to LXR β (Fig. 1D) resulted in the failure of LXR β -mediated transactivation. RC was shown to have no ability to activate PPAR γ , RAR α , RAR β , RAR γ , FXR, and RXR α (Fig. 1C).

In contrast to RC, RF was inactive for both LXR α and LXR β -mediated transactivation (Fig. 1B). This compound was able to bind to LXR α and LXR β (Fig. 2A) but was unable to promote coactivator association (Fig. 1C). Structural differences between RC and RF suggest that the C-14 hydroxyl group of RC plays a critical role in inducing coactivator association to LXR α , but not to LXR β , leading to transactivation in a subtype-selective manner.

The competition with the synthetic agonist TO-1317 for the coactivator association (Fig. 2A) and the receptor-mediated transactivation (Fig. 2B) demonstrates that RC functions as an LXR α partial agonist and an LXR β antagonist. RF functions as an antagonist of LXR α . RF decreased LXRE-dependent luciferase transcription in HepG2 cells to the level of endogenous-ligand depletion by compactin (Fig. 2C), suggesting that endogenous ligand-mediated LXR activation was inhibited by RF. RC increased LXRE-dependent transcription in compactin-treated cells, but not in the absence of compactin. In the intact cells, RC, as well as RF, might compete with endogenous ligands for LXR activation, thereby decreasing endogenous ligand-mediated activation. This latent activity of RC might compensate for this reduction. RC and RF also competed with TO-1317 for SREBP-1c expression in THP-1 cells.

The efflux of cellular cholesterol to HDL constitutes the first stage in the reverse cholesterol transport pathway and plays a critical role in modulating the progression of atherosclerosis [21]. ABCA1 and ABCG1 have been shown to mediate this process [22,23], and LXR α and LXR β play redundant roles in their expression [9,24]. RC functions as an LXR α partial agonist and LXR β antagonist. However, RC was shown to raise ABCA1 and ABCG1 mRNA levels and enhance cellular cholesterol efflux in THP-1 cells (Fig. 3A and B). Because human LXR α expression is autoregulated by LXR α itself [25], the stimulation of LXR α may have a stronger effect than that of LXR β . RC and TO-1317 at higher concentrations increased cholesterol efflux even in the absence of apoA-I. Because THP-1 cells express apoE [26] and ABCA1 at the basal level, increased expression of apoE by LXR activation [27], caveolin-1 [28], or ABCA1 [29] could lead to enhanced cholesterol release, even without apoA-I.

We found that RC did not affect SREBP-1c and ABCG1 expression in hepatoma HepG2 cells (Fig. 3C). This coincides with the apparent inability of RC to enhance LXRE-dependent transactivation in this cell line (Fig. 2C). A possible explanation for this inability is the low LXR α /LXR β ratio in HepG2 cells. Alternatively, as a partial LXR α agonist/LXR β antagonist, RC might compete with endogenous ligands produced via the cholesterol synthesis pathway. It is also possible that coactivator-corepressor(s) different from those employed in THP-1 cells might be active in this cell line. Further studies are required to elucidate the precise mechanisms of RC's selective actions. However, because synthetic LXR agonists have been shown to cause hypertriglyceridemia [5], LXR modulators that do not upregulate hepatic lipogenic genes may enable improved therapeutic strategies.

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References

- [1] Edwards, P.A., Kast, H.R. and Anisfeld, A.M. (2002) BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. *J. Lipid Res.* 43, 2–12.
- [2] Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R. and Mangelsdorf, D.J. (1996) An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature* 383, 728–731.
- [3] Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A. and Willson, T.M. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J. Biol. Chem.* 272, 3137–3140.
- [4] Repa, J.J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J.M., Shimomura, I., Shan, B., Brown, M.S., Goldstein, J.L. and Mangelsdorf, D.J. (2000) Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. *Genes Dev.* 14, 2819–2830.
- [5] Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D.J., Lustig, K.D. and Shan, B. (2000) Role of LXRs in control of lipogenesis. *Genes Dev.* 14, 2831–2838.
- [6] Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A.H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Kimura, S., Ishibashi, S. and Yamada, N. (2001) Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. *Mol. Cell Biol.* 21, 2991–3000.
- [7] Joseph, S.B., Laffitte, B.A., Patel, P.H., Watson, M.A., Matsukuma, K.E., Walczak, R., Collins, J.L., Osborne, T.F. and Tontonoz, P. (2002) Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *J. Biol. Chem.* 277, 11019–11025.
- [8] Costet, P., Luo, Y., Wang, N. and Tall, A.R. (2000) Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J. Biol. Chem.* 275, 28240–28245.
- [9] Venkateswaran, A., Repa, J.J., Lobaccaro, J.M., Bronson, A., Mangelsdorf, D.J. and Edwards, P.A. (2000) Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J. Biol. Chem.* 275, 14700–14707.
- [10] Lu, T.T., Repa, J.J. and Mangelsdorf, D.J. (2001) Orphan nuclear receptors as eLIXiRs and FiXeRs of sterol metabolism. *J. Biol. Chem.* 276, 37735–37738.
- [11] Alberti, S., Schuster, G., Parini, P., Felkamp, D., Diczfalusy, U., Rudling, M., Angelin, B., Bjorkhem, I., Pettersson, S. and Gustafsson, J.A. (2001) Hepatic cholesterol metabolism and resistance to dietary cholesterol in LXRbeta-deficient mice. *J. Clin. Invest.* 107, 565–573.
- [12] Peet, D.J., Turley, S.D., Ma, W., Janowski, B.A., Lobaccaro, J.M., Hammer, R.E. and Mangelsdorf, D.J. (1998) Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 93, 693–704.
- [13] Schuster, G.U., Parini, P., Wang, L., Alberti, S., Steffensen, K.R., Hansson, G.K., Angelin, B. and Gustafsson, J.A. (2002) Accumulation of foam cells in liver X receptor-deficient mice. *Circulation* 106, 1147–1153.
- [14] Yoshida, T., Hashimoto, T., Takaoka, S., Kan, Y., Tori, M. and Asakawa, Y. (1996) Phenolic constituents of the liverwort: four novel cyclic bis(benzyl) dimers from *Blasia pusilla* L. *Tetrahedron* 52, 14487–14500.
- [15] Hashimoto, T., Yoshida, T., Kan, Y., Takaoka, S., Tori, M. and Asakawa, Y. (1994) Structures of four novel macrocyclic bis(benzyl) dimers, pusilatins A–D from the liverwort *Blasia pusilla*. *Tetrahedron Lett.* 35, 909–910.
- [16] Nishimaki-Mogami, T., Ume, M., Fujino, T., Sato, Y., Tamehiro, N., Kawahara, Y., Shudo, K. and Inoue, K. (2004) Identification of intermediates in the bile acid synthetic pathway as ligands for the farnesoid X receptor. *J. Lipid Res.* 45, 1538–1545.

- [17] Fu, X., Menke, J.G., Chen, Y., Zhou, G., MacNaul, K.L., Wright, S.D., Sparrow, C.P. and Lund, E.G. (2001) 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. *J. Biol. Chem.* 276, 38378–38387.
- [18] Suzuki, S., Nishimaki-Mogami, T., Tamahiro, N., Inoue, K., Arakawa, R., Abe-Dohmae, S., Tanaka, A.R., Ueda, K. and Yokoyama, S. (2004) Verapamil increases the apolipoprotein-mediated release of cellular cholesterol by induction of ABCA1 expression via Liver X receptor-independent mechanism. *Arterioscler. Thromb. Vasc. Biol.* 24, 519–525.
- [19] DeBose-Boyd, R.A., Ou, J., Goldstein, J.L. and Brown, M.S. (2001) Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. *Proc. Natl. Acad. Sci. USA* 98, 1477–1482.
- [20] Wong, J., Quinn, C.M. and Brown, A.J. (2004) Statins inhibit synthesis of an oxysterol ligand for the liver x-receptor in human macrophages with consequences for cholesterol flux. *Arterioscler. Thromb. Vasc. Biol.* 24, 2365–2371.
- [21] Joyce, C., Freeman, L., Brewer Jr., H.B. and Santamarina-Fojo, S. (2003) Study of ABCA1 function in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* 23, 965–971.
- [22] Singaraja, R.R., Brunham, L.R., Visscher, H., Kastelein, J.J. and Hayden, M.R. (2003) Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. *Arterioscler. Thromb. Vasc. Biol.* 23, 1322–1332.
- [23] Wang, N., Lan, D., Chen, W., Matsuura, F. and Tall, A.R. (2004) ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc. Natl. Acad. Sci. USA* 101, 9774–9779.
- [24] Repa, J.J., Turley, S.D., Lobaccaro, J.A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R.A., Dietschy, J.M. and Mangelsdorf, D.J. (2000) Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science* 289, 1524–1529.
- [25] Laffitte, B.A., Joseph, S.B., Walczak, R., Pei, L., Wilpitz, D.C., Collins, J.L. and Tontonoz, P. (2001) Autoregulation of the human liver X receptor alpha promoter. *Mol. Cell Biol.* 21, 7558–7568.
- [26] Tajima, S., Hayashi, R., Tsuchiya, S., Miyake, Y. and Yamamoto, A. (1985) Cells of a human monocytic leukemia cell line (THP-1) synthesize and secrete apolipoprotein E and lipoprotein lipase. *Biochem. Biophys. Res. Commun.* 126, 526–531.
- [27] Laffitte, B.A., Repa, J.J., Joseph, S.B., Wilpitz, D.C., Kast, H.R., Mangelsdorf, D.J. and Tontonoz, P. (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl. Acad. Sci. USA* 98, 507–512.
- [28] Arakawa, R., Abe-Dohmae, S., Asai, M., Ito, J.I. and Yokoyama, S. (2000) Involvement of caveolin-1 in cholesterol enrichment of high density lipoprotein during its assembly by apolipoprotein and THP-1 cells. *J. Lipid Res.* 41, 1952–1962.
- [29] Abe-Dohmae, S., Ikeda, Y., Matsuo, M., Hayashi, M., Okuhira, K., Ueda, K. and Yokoyama, S. (2004) Human ABCA7 supports apolipoprotein-mediated release of cellular cholesterol and phospholipid to generate high density lipoprotein. *J. Biol. Chem.* 279, 604–611.

Thyroid Hormone Targets Matrix Gla Protein Gene Associated With Vascular Smooth Muscle Calcification

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Thyroid Hormone Targets Matrix Gla Protein Gene Associated With Vascular Smooth Muscle Calcification

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Abstract—Thyroid hormones have marked cardiovascular effects in vivo. However, their direct effects on vascular smooth muscle cells have been unclear. Because thyroid hormones play critical roles in bone remodeling, we hypothesized that they are also associated with vascular smooth muscle calcification, one of the pathological features of vascular sclerosis. To test this hypothesis, we examined the effects of 3',3,5-triiodo-L-thyronine (T_3) on the expression of calcification-associated genes in rat aortic smooth muscle cells (RAOSMCs). Quantitative RT-PCRs revealed that a physiological concentration of T_3 (15 pmol/L free T_3) increased mRNA level of matrix Gla protein (MGP), which acts as a potent inhibitor of vascular calcification in vivo, by 3-fold in RAOSMCs, as well as in cultured human coronary artery smooth muscle cells. In RAOSMCs transiently transfected with a luciferase reporter gene driven by the MGP promoter, T_3 significantly stimulated luciferase activity. In addition, RNA interference against thyroid hormone receptor- α gene diminished the effect of T_3 on MGP expression. Aortic smooth muscle tissues from methimazole-induced hypothyroid rats (400 mg/L drinking water; 4 weeks) also showed a 68% decrease in the MGP mRNA level, as well as a 33% increase in calcium content compared with that from the control euthyroid animals, whereas hyperthyroidism (0.2 mg T_3 /kg IP; 10 days) upregulated MGP mRNA by 4.5-fold and reduced calcium content by 11%. Our findings suggest that a physiological concentration of thyroid hormone directly facilitates MGP gene expression in smooth muscle cells via thyroid hormone nuclear receptors, leading to prevention of vascular calcification in vivo. (*Circ Res.* 2005;97:550-557.)

Key Words: calcium ■ gene expression ■ nuclear receptors ■ vascular smooth muscle ■ thyroid hormone

Thyroid hormone has marked effects on differentiation, development, and metabolic balance of virtually every body tissue. The action of thyroid hormone is mediated by high-affinity thyroid hormone nuclear receptors (TRs), which recognize specific response elements in the promoters of target genes and regulate their transcriptional activity in response to the hormone. Alterations in thyroid hormone levels have a profound impact on the cardiovascular system, which include changes in myocardial contractility, heart rate, and resistance of peripheral vasculature. Hyperthyroidism leads to positive inotropic, lusitropic, and chronotropic effects on the heart and low systemic vascular resistance, whereas the opposite is observed in hypothyroidism. In myocardium, the mechanisms for these changes are based on altered expression levels of several key proteins involved in the regulation of intracellular ion homeostasis. The effects of thyroid hormone on cardiac contractility as well as rates of contraction and relaxation are mainly mediated by increases in the levels of the sarcoplasmic reticulum Ca^{2+} -ATPase and decreases in its inhibitor phospholamban in cardiomyocytes.¹ The positive chronotropic effect of thyroid hormone is associated with altered expression levels in plasmalemmal ion

channels/transporters in the heart, such as Kv1.5, Kv4.2, minK, hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2), HCN4, Na^+ - Ca^{2+} exchanger, and Na^+ - K^+ -ATPase.²⁻⁵ In contrast, although $\approx 25\%$ of hypothyroid patients have diastolic hypertension,⁶ the mechanism for the altered systemic vascular resistance under an abnormal thyroid hormone status is not well understood. To date, a loss of nongenomic vasodilating action of thyroid hormone⁷ and atherosclerosis attributable to hypercholesterolemia⁸ have been associated with the increased systemic vascular resistance under hypothyroidism.⁹ Recently, mRNAs for TR isoforms were identified in aortic and coronary smooth muscle cells, suggesting that a direct genomic action of thyroid hormone may play a significant role in vascular smooth muscle.¹⁰ Although extremely high concentrations of thyroid hormone are known to regulate expression of several genes in vascular smooth muscle cells,^{10,11} the physiological and direct target genes of thyroid hormone in vascular smooth muscle cells are not known.⁹

Arterial calcification is a common pathological feature of vascular sclerosis, as well as a variety of metabolic disorders such as diabetes and renal disease. Decades ago, cretinism

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was found to be associated with arterial calcification, especially when patients did not receive sufficient thyroid hormone replacement therapy.¹² However, the mechanism for the calcification in cretins has been to date unclear. A subset of vascular smooth muscle cells, named "calcifying vascular cells," was demonstrated recently to undergo osteogenic and chondrogenic differentiation in culture, indicating that some vascular smooth muscle cells still have the potential for multiple lineages.¹³ Because thyroid hormone plays a critical role in bone remodeling,¹⁴ we hypothesized that thyroid hormone is also associated with vascular calcification. To test this hypothesis, we investigated the effect of thyroid hormone on vascular smooth muscle calcification and expression profiles of calcification-associated genes *in vitro* and *in vivo*, and identified matrix Gla protein (MGP) gene as a target of thyroid hormone in vascular smooth muscle cells.

Materials and Methods

Cell Culture

α -Actin-positive rat aortic smooth muscle cells (RAOSMCs) were obtained from Cell Applications, Inc. and were cultured on 6-well cell culture plates at 37°C in a humidified atmosphere of 95% air/5% CO₂ in growth medium (GM; Dulbecco's Minimal Essential Medium [DMEM] supplemented with 10% FCS, 100 U/mL penicillin, and 100 μ g/mL streptomycin). Cells up to passage 5 were used for the experiments. The culture media were changed every 48 hours. Thyroid hormone-depleted serum was prepared as described previously.¹⁵ To evaluate effects of thyroid hormone on gene expression profiles of the synthetic phenotype of RAOSMCs, the cells at 50% of confluence were cultured in thyroid hormone-depleted medium (TDM; DMEM containing 10% thyroid hormone-depleted serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin) for 2 days and stimulated with 3',3,5-triiodo-L-thyronine (T₃) for another 2 days. The contractile form of RAOSMCs was obtained by culturing confluent cells in serum-free differentiation medium (DM; DMEM supplemented with 1 \times ITS-X (Invitrogen), 5 mmol/L taurine, 100 U/mL penicillin, and 100 μ g/mL streptomycin) for 10 days, followed by T₃ treatment for 2 days. The transition of the cell phenotype in DM was confirmed by immunoblotting for nonmuscle myosin heavy chain (SMemb) and smooth muscle myosin heavy chain-2 (SM2). To examine effects of thyroid hormone on calcium accumulation, confluent RAOSMCs were cultured as described previously¹⁶ in TDM with or without 100 ng/mL recombinant human bone morphogenic protein-2 (rBMP2; R & D Systems) in a cell culture dish with or without collagen type IV (Col4) coating (BD Biosciences). Supplementation with β -glycerophosphate, which facilitates smooth muscle cell calcification,¹⁷ was omitted because of a decrease in the signal-to-background ratio. Cells were subsequently stimulated with T₃ for 5 days. The concentrations of free T₃ (fT₃) and free L-thyroxine (T₄) in the serum-containing medium were determined using chemiluminescent enzyme immunoassay at a clinical diagnostic laboratory. The detection limits for fT₃ and fT₄ were 1.1 pmol/L and 1.7 pmol/L, respectively. Human coronary artery smooth muscle cells (HCASMCs; Cell Applications, Inc.) were maintained and transformed into the contractile phenotype before treatment with T₃ by culturing for 7 days in serum-free HCASMC DM (311D-500; Cell Applications, Inc) according to manufacturer instructions.

Animals

Male Sprague-Dawley rats (Japan SLC; Shizuoka, Japan) were maintained on rodent chow (Certified diet MF; Oriental Yeast, Co) and given water *ad libitum*. For generation of hypothyroid animals, methimazole (MMI; 400 mg/L) was added to the drinking water for 4 weeks. Hyperthyroid rats were generated by daily injection of T₃ (0.2 mg/kg body weight IP) for 10 days. Plasma concentrations of

fT₃ and fT₄ were measured, as described above. After the treatment with MMI or T₃, the thoracic aorta was isolated. Animals were 12 weeks old when killed. Aortic smooth muscle tissue for measurements of calcium accumulation and gene expression was cleared of fat, connective tissue, and an endothelium and stored at -80°C until use. For pathological examination, the aortic tissue was fixed in 10% formaldehyde. Transverse aortic sections were taken from the fixed tissue and stained with hematoxylin and eosin. All animals were treated in accordance with laboratory animal care guidelines of National Institute of Health Sciences at Tokyo.

Calcium Accumulation

Calcium content in RAOSMCs and rat aortic smooth muscle tissues were determined as described previously¹⁶ using *o*-cresolphthalein complexone method. Protein concentration was determined using Bio-Rad protein assay reagent and BSA as a standard.

Real-Time Quantitative RT-PCR

Total RNA was isolated from smooth muscle cells and tissues using Sepasol reagent (Nakalai Tesque) containing 0.1 mg/mL glycogen (Roche Diagnostics) and was treated with DNaseI (Promega) according to the manufacturer protocols. To quantitate specific mRNA levels, the real-time progress of target sequence-specific amplification was monitored during RT-PCR using TaqMan chemistry and PRISM7000 Sequence Detection System (Applied Biosystems). An 18S ribosomal RNA was used as an internal control for each RNA level. Sequences of the primers and the TaqMan probes are listed in supplemental Table I (available online at <http://circres.ahajournals.org>).

Western Blot Analysis

RAOSMCs and aortic smooth muscle tissues were homogenized in lysis buffer as described previously.¹⁸ After measuring protein concentrations, proteins were separated by SDS-PAGE and blotted onto polyvinylidene fluoride membranes, which were incubated with anti-SMemb or anti-SM2 monoclonal antibodies (Yamasa) or an anti-MGP polyclonal antibody (TransGenic) for 1 hour at room temperature. Subsequently, membranes were incubated with the secondary antibody conjugated with horseradish peroxidase for 1 hour. Signals were visualized and quantified using ECL Plus system (Amersham Biosciences) and LAS-3000 Imaging System (FUJIFILM), respectively.

Promoter Activity Assay

Fragments between -1752 and -1 of 5' flanking sequence of the MGP gene exon 1 and between -1895 and -1 of 5' flanking sequence of the stanniocalcin-1 (STC1) gene exon 1 were amplified using rat tail genomic DNA as a template. The primers for PCR amplifications were designed as based on the nucleotide sequences (MGP forward: CAAGGGTACCGGTTTGAGAGACCACGAGAC; MGP reverse: CTTGAAGCTTCTCTGTGAGTCTGCCTCTGTG; STC1 forward: CAAGCTCGAGCCCCTGATATTTCAGCATGG; STC1 reverse: CTTGAAGCTTAGGTGAGGATTGAGGAGG). The amplicons were subcloned into the firefly luciferase expression vector pGL3-Basic (Promega). RAOSMCs in the contractile state, grown on a 24-well plate, were transiently cotransfected with 225 ng/well of each promoter luciferase plasmid and 75 ng/well of pRL-TK control plasmid (Promega) using FuGene6 (Roche). Three hours after transfection, cells were incubated with or without T₃. The luciferase activity was defined as a ratio of the firefly luciferase signal to the renilla luciferase signal, which was measured with Dual-Luciferase Reagent (Promega). The transfection efficiency of the plasmids was estimated to be 1% to 10% of the total RAOSMCs, as assessed by transfection experiments with an enhanced green fluorescent protein expression vector pEGFP-N1 (BD Biosciences Clontech; data not shown).

RNA Interference Against TR α

RAOSMCs in the synthetic form were transiently transfected with StealthRNAi (Invitrogen) specific for TR α gene (sense: CCAGAA-GAACCUCUAUCCACCUAU; antisense: AUAGGUGGGAUG-

Free Thyroid Hormone Concentrations in Rat Plasma and Serum-Containing Cell Culture Medium

Plasma/Medium	fT ₃ (pmol/L)	fT ₄ (pmol/L)	Calculated Range of Thyroid Hormone Activity (pmol fT ₃ /L)
Plasma from a euthyroid rat	4.2±0.6	27±2	6.9–31
Plasma from a hypothyroid rat (MMI treatment for 1 month)	<1.1	<1.8	<1.3–2.9
GM (DMEM, 10% FCS, P/S)	3.7±0.2	15±0.0	5.2–19
TDM (DMEM, 10% thyroid hormone–depleted FCS, P/S)	<1.1	2.7±0.2	<1.4–3.8
TDM (DMEM, 10% thyroid hormone–depleted FCS, P/S) + 1 nmol/L T ₃	15±1*	3.4±0.1*	15–18

The range of thyroid hormone activity was calculated assuming that X% (X=0–100) of T₄ is processed to T₃ at the site of action in vivo and that the affinity of T₄ for TRs is one tenth that of T₃.

P/S indicates penicillin and streptomycin.

Values are means±SD; *P<0.05 vs TDM without T₃ supplementation (Student *t* test).

GAGGUUCUUCUGG) or StealthRNAi negative control medium GC (Invitrogen) using Lipofectamine 2000 (Invitrogen), according to manufacturer instructions and incubated in TDM for 2 days. Cells were then treated with or without T₃ (15 pmol/L fT₃) for another 2 days. Total RNA was isolated from cells before and after T₃ treatment for mRNA determinations.

Statistics

Data were expressed as means±SEM unless otherwise indicated. Data were analyzed for statistical significance by Student *t* test or ANOVA with Student-Newman-Keuls test as a post hoc test. Significance was imparted at the P<0.05 level.

Results

Free Thyroid Hormone Concentrations

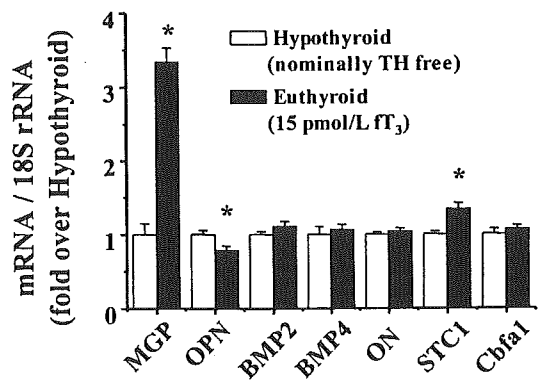
As shown in the Table, plasma concentrations of fT₃ and fT₄ in euthyroid rats were 4.2±0.6 pmol/L and 27±2 pmol/L (mean±SD), respectively. The MMI treatment decreased fT₃ and fT₄ to <1.1 pmol/L and to <1.8 pmol/L, respectively, indicating that the animals were hypothyroid. The fT₃ and fT₄ levels in GM containing 10% FCS were 3.7±0.2 pmol/L and 15±0.0 pmol/L (mean±SD), respectively, and happened to be similar to those in plasma from euthyroid rats. The free concentrations of thyroid hormones in TDM (<1.1 pmol/L fT₃; 2.7 pmol/L fT₄) were low enough to keep TRs inactivated because the K_d values of T₃ for TRs are known to be ≈10 to 100 pmol/L¹⁹ and because T₄ has ≈10-fold lower affinity for TRs than that of T₃. In vascular smooth muscle cells, T₄ is known to be converted to T₃ by type II iodothyronine deiodinase,¹⁰ although the conversion rate in vivo is not clear. Assuming that X% (X=0 to 100) of T₄ is processed to T₃ at the site of action in vivo and that the affinity of T₄ for TRs is one tenth of that of T₃, the total activity of thyroid hormones in euthyroid rat plasma should be equivalent to that of 6.9 to 31 pmol/L [4.2+27×X/100+27×(1–X/100)/10] of fT₃ alone. The supplementation of T₃ to TDM at 1 nmol/L of total concentration resulted in an increase in fT₃ to 15±1 pmol/L (mean±SD), with a slight increase in fT₄, therefore, it was regarded within a euthyroid range.

T₃-Induced Gene Expression and Calcification in Cultured RAOSMCs

Vascular smooth muscle cells show a high degree of plasticity and are able to interchange between a differentiated, contractile phenotype and a proliferating, synthetic phenotype. Therefore, we first examined the effects of T₃ on expression profiles of calcification-associated genes in both

phenotypes. RAOSMCs in GM predominantly expressed a marker of synthetic phenotype: SMemb. The replacement of the medium with DM reduced the protein level of SMemb to 28% and increased SM2 expression by 11.6-fold (online Figure I), indicating the transition of the phenotype. In the synthetic phenotype, T₃ (1 nmol/L total T₃=15 pmol/L fT₃) led to upregulation of mRNAs for MGP and STC1×3.3-fold and 1.3-fold, respectively (Figure 1A), whereas osteopontin

A



B

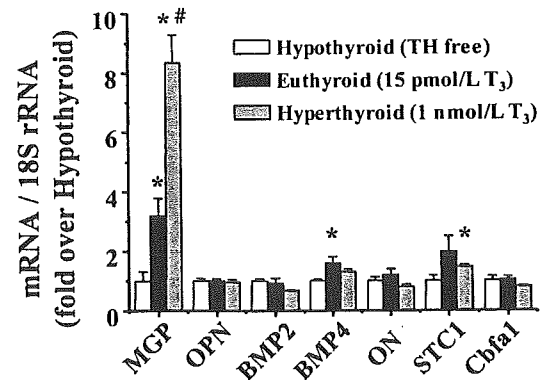


Figure 1. T₃ regulated expression of calcification-associated genes in cultured RAOSMCs in synthetic and contractile forms. A, Effect of T₃ on mRNA expression of calcification-associated genes in the synthetic form of RAOSMCs cultured in TDM. Cells were treated with 15 pmol/L fT₃ for 2 days. B, Effect of T₃ on mRNA expression of calcification-associated genes in the contractile form of RAOSMCs cultured in the DM. Cells were treated with 15 pmol/L or 1 nmol/L T₃ for 2 days. Values are expressed as means±SEM (n=4) TH indicates thyroid hormone. *P<0.05 vs hypothyroid; #P<0.05 vs euthyroid.

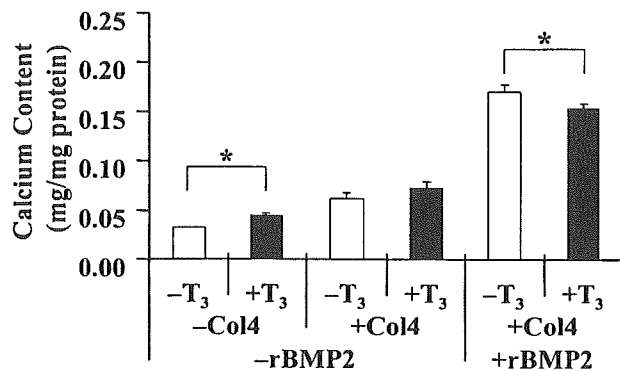


Figure 2. Effects of T₃ on calcium accumulation in cultured RAOSMCs. The confluent cells cultured in TDM were stimulated by 15 pmol/L fT₃. In the absence of rBMP2, T₃ significantly increased calcium content in RAOSMCs cultured in noncoated vessels. Supplementation with rBMP2 increased the basal calcium content in RAOSMCs in Col4-coated vessels and reversed the effect of T₃ on the calcification. Values are expressed as means±SEM (n=6 to 8); *P<0.05.

(OPN) was downregulated by 21%. The mRNA levels of BMP2, bone morphogenic protein-4 (BMP4), osteonectin (ON), and core binding factor α 1 (Cbfa1; also known as Runx2 or Osf2) were not significantly altered by the T₃ treatment. Specific signals for osteocalcin and bone sialoprotein mRNA were not detected by several independent primer sets in all experiments of the present study, presumably because of their low abundance in vascular smooth muscle cells. In the contractile phenotype in DM, T₃ (15 pmol/L) induced upregulation of MGP and BMP4×3.2-fold and 1.6-fold, respectively (Figure 1B). A higher concentration of T₃ (1 nmol/L) resulted in a further increase in the MGP mRNA level and a significant upregulation of STC1 mRNA, indicating the dose dependency of the effects. Messenger RNA levels of OPN, BMP2, ON, STC1, and Cbfa1 in the contractile phenotype were not significantly altered by the T₃ treatment. Among the calcification-associated genes, MGP and STC1 genes were commonly upregulated by T₃ in both phenotypes, suggesting that these two genes were relatively essential in RAOSMCs as targets of thyroid hormone.

Because the effect of MGP on calcification and osteogenic differentiation of vascular smooth muscle cells depends on availability of BMP2 and Col4,^{20,21} cell calcification was determined in the absence or presence of these factors. In the absence of rBMP2 and Col4 coating, treatment with T₃ (1 nmol/L total T₃=15 pmol/L fT₃) for 5 days resulted in an increase in calcium content by 39% in RAOSMCs (Figure 2). The same treatment tended to have the similar effect on cells in a Col4-coated vessel in the absence of rBMP2. In contrast, T₃ led to a significant decrease in cellular calcium by 10% in the presence of rBMP2 and Col4-coating.

Transcriptional Regulation of MGP and STC1 Genes by T₃

To test a hypothesis that the promoters of MGP and STC1 genes were under regulation of thyroid hormone, RAOSMCs were transiently transfected with luciferase reporters under control of the MGP and STC1 promoters. The 5' flanking sequences of MGP and STC1 genes have been submitted to

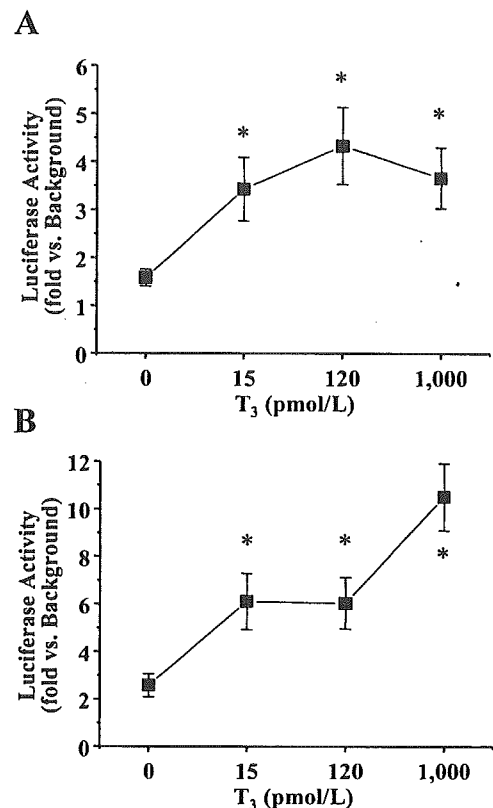


Figure 3. T₃ activated luciferase reporter genes driven by rat MGP (A) and STC1 (B) promoters in the contractile form of RAOSMCs. Cells were transiently cotransfected with a reporter plasmid containing a promoter region of rat MGP or STC1 gene and an internal control plasmid (pRL-tk) and were treated with T₃ for 2 days. The cell extracts were assayed for luciferase activity. Values are normalized to the background (luciferase activity of the cells transfected with promoterless pGL3-Basic vector and pRL-tk) and expressed as means±SEM (n=6); *P<0.05 vs control.

NCBI (accession numbers AY750958 and AY750959, respectively). Transcription Element Search System (TESS)²² revealed that consensus sequences of the thyroid hormone response element were located in 585 to 600 (TGTC-CCCAATGAACC) and 1009 to 1024 (TGGAGACAGGAG-GACA) bases upstream of the putative transcription initiation sites of MGP and STC1 genes, respectively. Treatments of the cells with T₃ (15 pmol/L to 1 nmol/L) for 48 hours resulted in significant increases in transcriptional activity compared with vehicle treatment (Figure 3).

Regulation of MGP and STC1 Expression via TRs

Arterial smooth muscle cells express TR α 1 and TR α 2 nuclear receptor isoforms strongly and TR β 1 and TR β 2 relatively weakly.¹⁰ To evaluate the involvement of TRs in the T₃-induced upregulation of MGP and STC1 mRNAs, RNA interference (RNAi) was performed using StealthRNAi specific for the TR α gene encoding TR α 1 and TR α 2. The RNAi in RAOSMCs for 2 days significantly attenuated mRNA expression of TR α 1 and TR α 2 by 66% and 57%, respectively, compared with the controls (Figure 4). The RNAi was also associated with upregulation of MGP mRNA by 32%

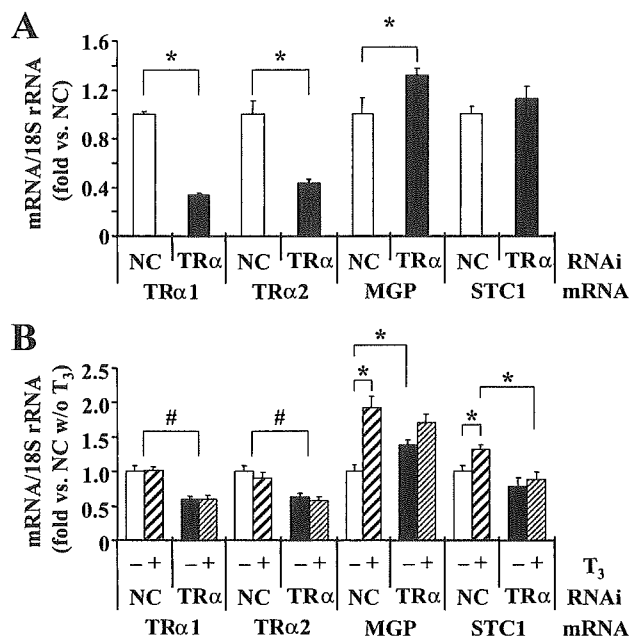


Figure 4. The RNAi against TR α gene in RAOSMCs (synthetic form). A, RNAi against TR α gene for 2 days led to significant decreases in expression of TR α 1 and TR α 2 to the same extent and a slight increase in the MGP expression. B, The expression of TR α 1 and TR α 2 remained reduced after the following incubation for 2 more days and was not altered by T₃ (15 pmol/L fT₃). The RNAi was associated with loss of responsiveness of MGP and STC1 mRNA to T₃. Values are expressed as means \pm SEM (n=4 to 6 [A] and 8 [B]). NC indicates negative control. **P*<0.05 (Student *t* test [A] or Student-Newman-Keuls test [B]); #*P*<0.05 association with RNAi (two-way ANOVA).

compared with the controls, suggesting that unliganded TR α 1 or TR α 2, which has no affinity for thyroid hormones, had an inhibitory effect on the expression of MGP. The mRNA level of STC1 was not altered by the RNAi. Incubation with 15 pmol/L fT₃ for the following 2 days facilitated the expression of MGP and STC1 in the control group, whereas no significant effect of T₃ on MGP and STC1 gene expression was detected in the RNAi group. T₃ had no effect on the expression of the TR α isoforms.

Calcium Accumulation in Hypothyroid Rat Aorta

As examined by hematoxylin-eosin staining, the cross-sections of aorta from rats treated with MMI for 4 weeks did not show obvious calcified foci (Figure 5A). However, *o*-cresolphthalein complexone experiments indicated that the 4-week treatment with MMI significantly increased the calcium content in the rat aortic smooth muscle tissues by 33% compared with that of euthyroid animals (Figure 5B). Quantitative RT-PCRs revealed that mRNA levels of MGP, BMP4, ON, and Cbfa1 were downregulated by 68%, 87%, 69%, and 72%, respectively, by the MMI treatment. OPN, BMP2, and STC1 mRNA levels were not significantly altered (Figure 5C). MMI also attenuated protein expression of MGP by 54% (Figure 5D). Calcified foci were not observed even in aortic cross-sections from rats treated with MMI for 12 weeks (online Figure II), suggesting that the calcification was not progressive.

Calcium Content in Hyperthyroid Rat Aorta

Hyperthyroidism elicits pronounced vascular relaxation.⁹ However, the effect of hyperthyroidism on vascular calcification has been unclear. Therefore, it is of interest to compare effects of hyperthyroidism on the expression profiles of calcification-associated genes with those of hypothyroidism. In contrast to the aortic smooth muscle from hypothyroid rats, daily injections of T₃ for 10 days led to a decrease in the calcium content in the rat aortic smooth muscle tissues by 11% compared with that of euthyroid animals (Figure 6A). Quantitative RT-PCRs showed that mRNA levels of MGP, OPN, and BMP2 were upregulated by 4.5-fold, 4.9-fold, and 3.4-fold, respectively, by the T₃ treatment, whereas hyperthyroidism resulted in a significant decrease in the level of STC1 mRNA (Figure 6B).

Upregulation of MGP mRNA in Cultured HCASMCs

To demonstrate that thyroid hormone regulates MGP gene expression in vascular smooth muscle cells of a different species, we determined MGP mRNA levels in HCASMCs in the presence and absence of T₃. Treatment with a physiological concentration (15 pmol/L) of T₃ for 2 days led to a significant increase in MGP mRNA by 40% (1.0 \pm 0.07 [hypothyroid 0 pmol/L T₃] versus 1.40 \pm 0.13 [euthyroid 15 pmol/L T₃] in an arbitrary unit; n=12).

Discussion

In the present study, thyroid hormone led to an upregulation of MGP in arterial smooth muscle cells in vitro regardless of culture condition, phenotype, and animal species of the cells. The transcriptional activity of the MGP gene was increased by T₃, and reduction of TR α gene expression led to a loss of responsiveness of the MGP gene to T₃, suggesting that the effect of T₃ is based on a genomic action via TR α 1. Furthermore, in vivo hormone levels were positively and negatively associated with the expression of MGP and calcification in vascular smooth muscle, respectively. Because aortic smooth muscle from hypothyroid rats showed no obvious neointimal formation, the vascular calcification under the hypothyroidism is likely to be similar to that of media sclerosis. In aortic smooth muscle from hypothyroid rats, expression levels of calcification activators BMP4, ON, and Cbfa1 were decreased, whereas hyperthyroidism upregulated another calcification activator BMP2. However, calcium content in aortic smooth muscle was increased in hypothyroidism, and the opposite was observed in hyperthyroidism, suggesting that the expression or function of calcification inhibitors, such as MGP and OPN, are more dominant for the phenotypic outcome in vivo, compared with those of the calcification activators.

MGP is a mineral-binding extracellular matrix protein synthesized by vascular smooth muscle cells and chondrocytes. Luo et al have shown that ablation of MGP gene in mice causes extensive and lethal calcification and cartilaginous metaplasia of the media of all elastic arteries, indicating that MGP has an inhibitory effect on media calcification in vivo.²³ In contrast, in the same study, morphological analysis showed that heterozygous MGP knockout mice, which had a

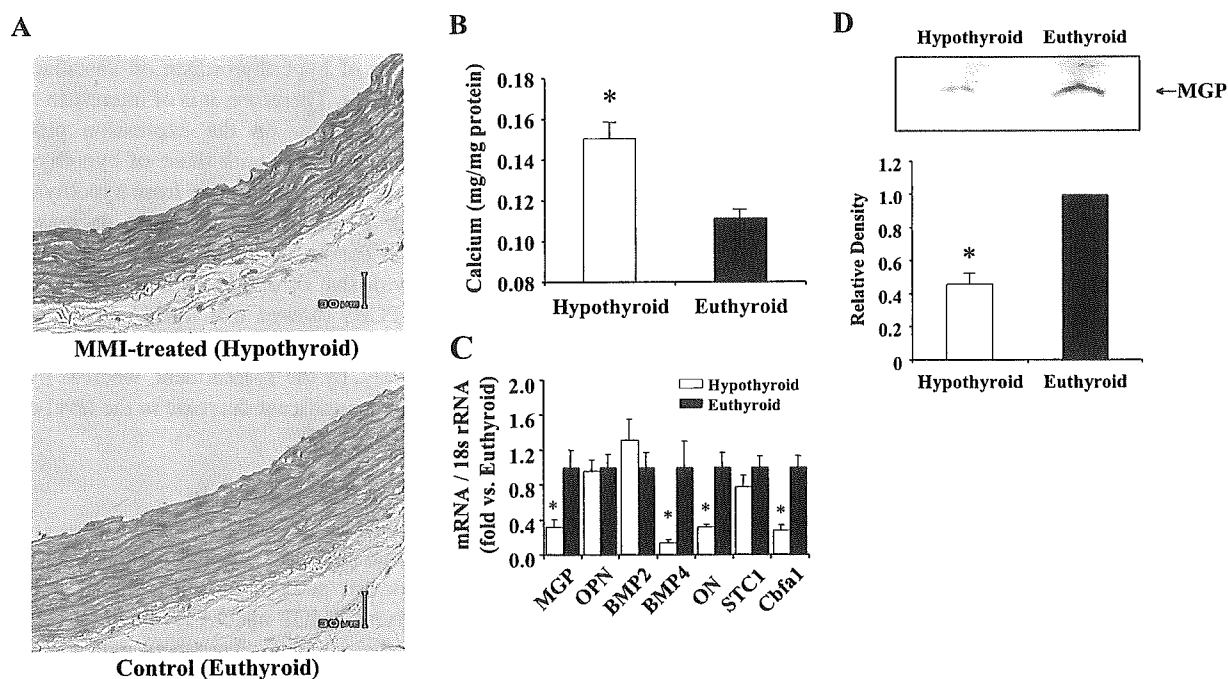


Figure 5. Hypothyroidism led to calcium accumulation in aortic smooth muscle and an altered expression profile of calcification-associated genes. Hypothyroidism was achieved by treatment of the animals with MMI (400 mg/L drinking water) for 4 weeks. A, Hematoxylin-eosin staining of cross-sections of aortae from euthyroid and hypothyroid rats. No obvious calcified foci were observed in either section. Bar=30 μ m. B, Effect of the hypothyroidism on calcium deposition in rat aortic smooth muscle. C, Effect of hypothyroidism on mRNA expression of calcification-associated genes in rat aortic smooth muscle. D, Effect of hypothyroidism on MGP protein expression in rat aortic smooth muscle. Values are expressed as means \pm SEM (n=7 to 8); * P <0.05 vs euthyroid.

similar decrease in an arterial MGP level to that by the MMI treatment in the present study, had no obvious calcified foci in arterial smooth muscle. However, this does not necessarily imply that the heterozygous ablation of MGP gene does not affect calcium content in arteries because biochemical quantification for tissue calcium has not been performed. Thus, it is still possible that there is a dose-response relationship between MGP and media calcification, and that an \approx 50% reduction of MGP results in some increase in calcium content, although it may not be morphologically evident.

The extracellular environment around smooth muscle cells is known to determine a functional role of MGP. Namely, calcification of vascular smooth muscle cells in the presence of a relatively high concentration of BMP2 was inhibited by MGP, whereas calcification of vascular smooth muscle cells under a low concentration of BMP2 was stimulated by MGP.²⁰ Moreover, extracellular matrix proteins, especially Col4, have significant influence on MGP function and vascular calcification.²¹ In fact, the effect of T_3 on calcification of RAOSMCs was determined by these environmental factors. Therefore, these results suggest that T_3 regulates smooth muscle cell calcification, at least partly, by promoting MGP expression, although not only vascular cells but also migratory adventitial pericytic myofibroblasts and circulating skeletal progenitors may have some additional contributions to vascular calcification in vivo.²⁴

STC1 is a mammalian homolog of stanniocalcin, the fish calcium/phosphate-regulating polypeptide that inhibits calcium flux into cells and stimulates phosphate reabsorption. In the present study, gene transcription of STC1 appeared to be

regulated by T_3 via $TR\alpha$ in a dose-dependent manner. However, the highest mRNA expression of STC1 tended to be achieved at a euthyroid status in vitro and in vivo, and hyperthyroidism significantly attenuated the STC1 mRNA expression in vivo. Recently, STC1 was shown to accelerate osteoblast development in an autocrine/paracrine manner in cultured fetal rat calvaria cells.²⁵ Therefore, the downregulation of STC1 may also contribute to the low calcium content in aortic smooth muscle of hyperthyroid rats, although the mechanism that offsets the increase in the STC1 gene transcription remains to be elucidated.

OPN is known to inhibit or promote vascular smooth muscle calcification in vivo in a phosphorylation-dependent manner.²⁶ Therefore, the changes in its expression in the synthetic form of RAOSMCs and in smooth muscle tissue of hyperthyroid rats may be also associated with the effect of thyroid hormone on calcium accumulation. However, as shown in the in vitro and MMI experiments, a physiological concentration of thyroid hormone is unlikely to target OPN gene directly, at least in the contractile form of aortic smooth muscle cells.

BMP2 was upregulated by hyperthyroidism in vivo, whereas BMP4 was downregulated by MMI-induced hypothyroidism. BMP2 is known to antagonize the effect of MGP,²⁰ and BMP4 has been suggested to play a significant role as a cytokine, a growth factor or a media-calcification promoter in vascular lesions of calciphylaxis.²⁷ The mRNA levels of ON and Cbfa1 were also decreased in hypothyroid rat aortic smooth muscle. With the exception of BMP4, these changes in vivo did not follow on from the in vitro experiments,

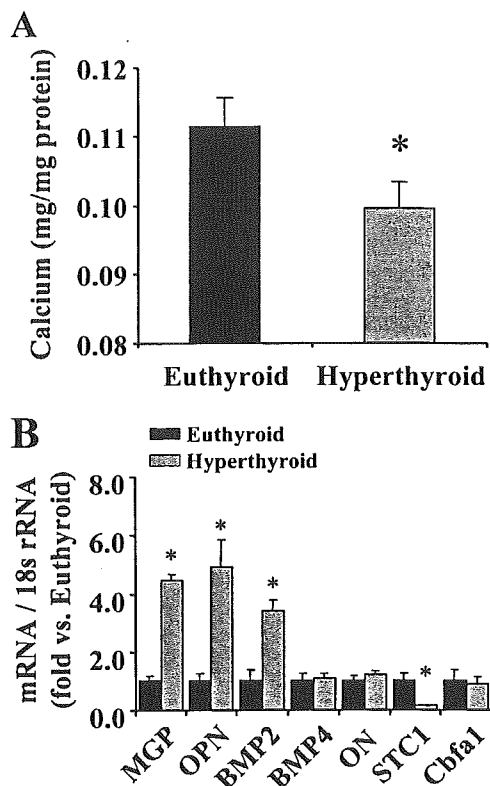


Figure 6. Hyperthyroidism decreased tissue calcium content in aortic smooth muscle and altered the expression profile of calcification-associated genes. Hyperthyroidism was achieved by daily injection of T_3 (0.2 mg/kg body weight IP) for 10 days. A, Effect of the hyperthyroidism on calcium deposition in rat aortic smooth muscle. C, Effect of hyperthyroidism on mRNA expression of calcification-associated genes in rat aortic smooth muscle. Values are expressed as means \pm SEM (n=7 to 8); * P <0.05 vs euthyroid.

suggesting that the alterations were not attributable to a direct effect of thyroid hormone on vascular smooth muscle cells. Because physiological concentrations of thyroid hormones upregulated BMP4 in the contractile form of RAOSMCs and in vivo (hypothyroid versus euthyroid), BMP4 may be another direct target of thyroid hormone in aortic smooth muscle cells. However, the expected influences of the changes in all the calcification activators above were apparently masked, aforementioned, indicating their minor roles in vascular calcification, compared with those of calcification inhibitors.

In summary, our findings, for the first time, demonstrate that a physiological concentration of thyroid hormone has significant genomic effects on vascular smooth muscle cells in vitro and in vivo, which are associated with vascular calcification. Most notably, a decrease in thyroid hormone and the concomitant increase in vascular calcification in vivo are marked by a decrease in the level of MGP expression, suggesting that a physiological concentration of thyroid hormone has a direct protective role against vascular smooth muscle calcification in vivo. Although vascular calcification has been thought to be benign, arterial calcification should alter vascular compliance. Thus, it is possible that the increased vascular stiffness underlies the high systemic vas-

cular resistance observed in hypothyroidism. Vascular calcification can lead to some other serious problems, including vascular stenosis, calciphylaxis, and even sudden death. Recently, a polymorphism in the promoter region of MGP gene was found to have a significant association with myocardial infarction in low-risk individuals and femoral calcification in the presence of atherosclerotic plaques, suggesting involvement of this mutation in coronary artery disease.²⁸ In addition, a recent meta-analysis of coronary artery calcium scores suggested that the calcium score is an independent risk factor to predict coronary heart disease events.²⁹ Therefore, further studies on the protective role of thyroid hormone and MGP against vascular smooth muscle calcification should provide insights into novel therapeutic strategies for the high systemic vascular resistance and blood pressure of hypothyroid patients, as well as for diseases associated with cardiovascular calcification such as diabetes, chronic renal insufficiency, and hypercholesterolemia.

Acknowledgments

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References

- Carr AN, Kranias EG. Thyroid hormone regulation of calcium cycling proteins. *Thyroid*. 2002;12:453–457.
- Abe A, Yamamoto T, Isome M, Ma M, Yaoita E, Kawasaki K, Kihara I, Aizawa Y. Thyroid hormone regulates expression of shaker-related potassium channel mRNA in rat heart. *Biochem Biophys Res Commun*. 1998;245:226–230.
- Gloss B, Trost S, Bluhm W, Swanson E, Clark R, Winkfein R, Janzen K, Giles W, Chassande O, Samarut J, Dillmann W. Cardiac ion channel expression and contractile function in mice with deletion of thyroid hormone receptor alpha or beta. *Endocrinology*. 2001;142:544–550.
- Boerth SR, Artman M. Thyroid hormone regulates Na^+ - Ca^{2+} exchanger expression during postnatal maturation and in adult rabbit ventricular myocardium. *Cardiovasc Res*. 1996;31:E145–E152.
- Orlowski J, Lingrel JB. Thyroid and glucocorticoid hormones regulate the expression of multiple Na,K-ATPase genes in cultured neonatal rat cardiac myocytes. *J Biol Chem*. 1990;265:3462–3470.
- Klein I, Ojamaa K. Thyroid hormone and the cardiovascular system. *N Engl J Med*. 2001;344:501–509.
- Ojamaa K, Klemperer JD, Klein I. Acute effects of thyroid hormone on vascular smooth muscle. *Thyroid*. 1996;6:505–512.
- Hak AE, Pols HAP, Visser TJ, Drexhage HA, Hofman A, Witteman JC. Subclinical hypothyroidism is an independent risk factor for atherosclerosis and myocardial infarction in elderly women: the Rotterdam Study. *Ann Intern Med*. 2000;132:270–278.
- Klein I, Ojamaa K. Thyroid hormone: targeting the vascular smooth muscle cell. *Circ Res*. 2001;88:260–261.
- Mizuma H, Murakami M, Mori M. Thyroid hormone activation in human vascular smooth muscle cells: expression of type II iodothyronine deiodinase. *Circ Res*. 2001;88:313–318.
- Fukuyama K, Ichiki T, Takeda K, Tokunou T, Iino N, Masuda S, Ishibashi M, Egashira K, Shimokawa H, Hirano K, Kanaide H, Takeshita A. Downregulation of vascular angiotensin II type 1 receptor by thyroid hormone. *Hypertension*. 2003;41:598–603.
- Komar NN, Gabrielsen TO. Arterial calcification in adult cretins. *Am J Roentgenol Radium Ther Nucl Med*. 1967;101:202–203.
- Tintut Y, Alfonso Z, Saini T, Radcliff K, Watson K, Bostrom K, Demer LL. Multilineage potential of cells from the artery wall. *Circulation*. 2003;108:2505–2510.

14. Bassett JH, Williams GR. The molecular actions of thyroid hormone in bone. *Trends Endocrinol Metab.* 2003;14:356–364.
15. Samuels HH, Stanley F, Casanova J. Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology.* 1979;105:80–85.
16. Jono S, Nishizawa Y, Shioi A, Morii H. Parathyroid hormone-related peptide as a local regulator of vascular calcification. Its inhibitory action on in vitro calcification by bovine vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1997;17:1135–1142.
17. Shioi A, Nishizawa Y, Jono S, Koyama H, Hosoi M, Morii H. Beta-glycerophosphate accelerates calcification in cultured bovine vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1995;15:2003–2009.
18. Sato Y, Ferguson DG, Sako H, Dorn GW II, Kadambi VJ, Yatani A, Hoit BD, Walsh RA, Kranias EG. Cardiac-specific overexpression of mouse cardiac calsequestrin is associated with depressed cardiovascular function and hypertrophy in transgenic mice. *J Biol Chem.* 1998;273:28470–28477.
19. Lazar MA, Chin WW. Nuclear thyroid hormone receptors. *J Clin Invest.* 1990;86:1777–1782.
20. Zebboudj AF, Shin V, Bostrom K. Matrix GLA protein and BMP-2 regulate osteoinduction in calcifying vascular cells. *J Cell Biochem.* 2003;90:756–765.
21. Shin V, Zebboudj AF, Bostrom K. Endothelial cells modulate osteogenesis in calcifying vascular cells. *J Vasc Res.* 2004;41:193–201.
22. Schug J, Overton GC. TESS: Transcription Element Search Software on the WWW. In: *Technical Report CBIL-TR-1997-1001-v0.0*, Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997:1–10.
23. Luo G, Ducey P, McKee MD, Pinero GJ, Loyer E, Behringer RR, Karsenty G. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature.* 1997;386:78–81.
24. Vattikuti R, Towler DA. Osteogenic regulation of vascular calcification: an early perspective. *Am J Physiol Endocrinol Metab.* 2004;286:E686–E696.
25. Yoshiko Y, Maeda N, Aubin JE. Stanniocalcin 1 stimulates osteoblast differentiation in rat calvaria cell cultures. *Endocrinology.* 2003;144:4134–4143.
26. Jono S, Peinado C, Giachelli CM. Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. *J Biol Chem.* 2000;275:20197–20203.
27. Griethe W, Schmitt R, Jurgensen JS, Bachmann S, Eckardt KU, Schindler R. Bone morphogenic protein-4 expression in vascular lesions of calciphylaxis. *J Nephrol.* 2003;16:728–732.
28. Herrmann SM, Whatling C, Brand E, Nicaud V, Garipey J, Simon A, Evans A, Ruidavets JB, Arveiler D, Luc G, Tiret L, Henney A, Cambien F. Polymorphisms of the human matrix gla protein (MGP) gene, vascular calcification, and myocardial infarction. *Arterioscler Thromb Vasc Biol.* 2000;20:2386–2393.
29. Pletcher MJ, Tice JA, Pignone M, Browner WS. Using the coronary artery calcium score to predict coronary heart disease events. A systematic review and meta-analysis. *Arch Intern Med.* 2004;164:1285–1292.

$G\alpha_{12/13}$ - and Reactive Oxygen Species-dependent Activation of c-Jun NH₂-terminal Kinase and p38 Mitogen-activated Protein Kinase by Angiotensin Receptor Stimulation in Rat Neonatal Cardiomyocytes*

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In the present study, we examined signal transduction mechanism of reactive oxygen species (ROS) production and the role of ROS in angiotensin II-induced activation of mitogen-activated protein kinases (MAPKs) in rat neonatal cardiomyocytes. Among three MAPKs, c-Jun NH₂-terminal kinase (JNK) and p38 MAPK required ROS production for activation, as an NADPH oxidase inhibitor, diphenyleneiodonium, inhibited the activation. The angiotensin II-induced activation of JNK and p38 MAPK was also inhibited by the expression of the $G\alpha_{12/13}$ -specific regulator of G protein signaling (RGS) domain, a specific inhibitor of $G\alpha_{12/13}$, but not by an RGS domain specific for $G\alpha_q$. Constitutively active $G\alpha_{12}$ or $G\alpha_{13}$ -induced activation of JNK and p38 MAPK, but not extracellular signal-regulated kinase (ERK), was inhibited by diphenyleneiodonium. Angiotensin II receptor stimulation rapidly activated $G\alpha_{13}$, which was completely inhibited by the $G\alpha_{12/13}$ -specific RGS domain. Furthermore, the $G\alpha_{12/13}$ -specific but not the $G\alpha_q$ -specific RGS domain inhibited angiotensin II-induced ROS production. Dominant negative Rac inhibited angiotensin II-stimulated ROS production, JNK activation, and p38 MAPK activation but did not affect ERK activation. Rac activation was mediated by Rho and Rho kinase, because Rac activation was inhibited by C3 toxin and a Rho kinase inhibitor, Y27632. Furthermore, angiotensin II-induced Rho activation was inhibited by $G\alpha_{12/13}$ -specific RGS domain but not dominant negative Rac. An inhibitor of epidermal growth factor receptor kinase AG1478 did not affect angiotensin II-induced JNK activation cascade. These results suggest that $G\alpha_{12/13}$ -mediated ROS production through Rho and Rac is essential for JNK and p38 MAPK activation.

Ang II¹ is a bioactive peptide involved in cardiac hypertrophy (1). Receptor stimulation by Ang II is assumed to activate G_q

and G_i, and turns on various signaling cascades dependent on cell types. Many groups have reported the regulation of MAPKs including JNK (2, 3), ERK (4, 5), and p38 MAPK (6) in a variety of cells (7). MAPKs are thought to be key intracellular transducers of mitogenic stimulation and have been implicated in the signaling pathways leading to cardiac hypertrophy (8, 9). An earlier study demonstrated that Ang II-induced JNK activation is dependent on extracellular calcium and protein kinase C and partially on a tyrosine kinase (2). On the other hand, Ang II-induced ERK activation is mediated by the Ras pathway or protein kinase C pathway (10). A recent study showed that β -arrestin-mediated internalization of ATR is involved in ERK activation (11). ERK or p38 MAPK activation by Ang II requires the EGF receptor transactivation, whereas JNK activation is regulated by other signaling proteins (7). These results indicate that the signal transduction mechanism of MAPK activation depends on the types of MAPKs and cellular contexts that are analyzed.

ROS such as hydrogen peroxide and oxygen radicals play various roles in living cells as a second messenger to elicit physiological responses or as a toxic intermediate leading to cellular damage (12). Recent studies suggest that ROS work as regulators of signal transduction (13, 14). We have reported that heterotrimeric G_{i/o} proteins are putative target molecules of ROS (15, 16). Although Ang II produces ROS in vascular smooth muscle cells and cardiac myocytes (6, 17, 18), the molecular mechanism of ROS production and the identification of ROS target molecules are largely unknown.

Rac, one of the small GTP-binding proteins, is believed to participate in the production of ROS by activating NADPH oxidase in neutrophil (19). Previous findings have demonstrated that one of the Rac effector PAKs mediates JNK activation by Ang II, and PAK phosphorylates a subunit of the NADPH oxidase complex (20). MAPKs are activated by Rac as

sin receptor; AT1R, angiotensin type 1 receptor; CA, constitutively active; DCF, 2',7'-dichlorofluorescein diacetate; DPI, diphenyleneiodonium; DN, dominant negative; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GRK2, G protein-coupled receptor kinase 2; GST, glutathione S-transferase; GST-TPR, GST fusion protein with TPR domain of protein phosphatase 5; JNK, c-Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; PAK, p21-activated kinase; Prx, peroxiredoxin; PTX, pertussis toxin; RGS, regulator of G protein signaling; ROCK, Rho-associated kinase; ROS, reactive oxygen species; TPR, tetratricopeptide repeat.

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¹ The abbreviations used are: Ang II, angiotensin II; ATR, angioten-

well as RhoA and Raf-1, and the resulting activation of MAPKs induces hypertrophic responses through the activation of intracellular signaling cascades (21–24). However, upstream molecules of and the relationship between these intracellular signaling molecules are not fully determined.

G $_{12}$ family G proteins, G $_{12}$ and G $_{13}$, couple with various G protein-coupled receptors and mediate physiological responses by interacting with different signaling proteins (25). The role of G $_{12/13}$ in the heart, however, has not been revealed because of the unavailability of a specific inhibitor. Recent studies showed that p115RhoGEF has an RGS domain for G $\alpha_{12/13}$ (26, 27). We examined by using the RGS domain of p115RhoGEF whether G $_{12/13}$ is involved in Ang II-mediated signal transduction pathway and ROS production and whether ROS work as a mediator in cardiac myocytes. To demonstrate the signal transduction cascade, we constructed various recombinant adenoviruses coding G $\alpha_{12/13}$ - or G α_q -specific RGS domains and DN-Rac. We demonstrate in the present study that Ang II-induced JNK and p38 MAPK activation requires ROS, and Ang II-induced ROS production is mediated by sequential activation of G $_{12/13}$, Rho, and Rac.

EXPERIMENTAL PROCEDURES

Materials and Plasmid Construction—AT1R blocker CV11974 was provided from Takeda Chemical Industries Ltd. (Osaka, Japan). AG1478, PTX, and Y27632 were purchased from Calbiochem. DPI, *N*-acetyl-L-cysteine, catalase, and PD123319 were from Sigma. Fura-2/AM was from Dojindo (Kumamoto, Japan). Collagenase and FuGENE 6 were from Roche Applied Science. 2',7'-Dichlorofluorescein diacetate was from Molecular Probe. Glutathione-Sepharose beads were from Amersham Biosciences. The cDNA coding DN-Rac1 was provided by Dr. Kozo Kaibuchi (Nagoya University, Nagoya, Japan). The plasmid coding PrxII was provided by Dr. Sue Goo Rhee (National Institutes of Health). The sequence coding Cdc42/Rac interactive binding domain of PAK was cloned from mouse brain, sequenced, and ligated into pGEX-4T-1 to make GST fusion protein construct. GST-TPR construct was provided by Dr. Manabu Negishi (Kyoto University, Kyoto, Japan). GST fusion proteins were expressed at room temperature and purified using glutathione-Sepharose as described (28, 29). Rat AT1R was cloned from rat heart, and the sequence was confirmed. Anti-phospho-ERK and anti-ERK antibodies were purchased from Cell Signaling. Anti-phospho-p38 MAPK and anti-p38 MAPK antibodies were from Promega. Anti-G α_{13} , anti-RhoA, horseradish peroxidase-conjugated anti-rabbit IgG, and anti-mouse IgG antibodies were from Santa Cruz Biotechnology. Anti-Rac1 antibody was from Transduction Laboratories.

Production of Adenoviruses—Recombinant adenoviruses of DN-Rac, PrxII, p115-RGS (the RGS domain of p115RhoGEF), GRK2-RGS, C3 toxin, CA-G α_q (R183C), CA-G α_{12} (Q229L), and CA-G α_{13} (Q226L) were produced by the method of He *et al.* (30) with a slight modification. Recombinant adenoviruses that do not express green fluorescent protein were also produced for DN-Rac, PrxII, hemagglutinin-tagged p115-RGS, and hemagglutinin-tagged GRK2-RGS. Adenovirus expressing GRK2-ct (the carboxyl-terminal domain of GRK2 that sequesters β subunits) was produced by the method as described (31). Adenovirus of LacZ was obtained from the RIKEN DNA Bank (Tsukuba, Japan).

Determination of JNK, ERK, and p38 MAPK Activation—The rat neonatal cardiac myocytes were isolated from 1–2-day-old Sprague-Dawley rat pups as previously described (15). The activity of JNK was determined as described (32, 33). ERK and p38 MAPK activation were determined by Western blot analysis using phospho-specific antibodies (32, 33). The optical density of the film was scanned and measured with Scion Image Software.

Quantification of Intracellular ROS Produced by Ang II Receptor Stimulation—ROS production was measured with the fluorescent dye DCF as described (34). The cardiomyocytes were plated on glass-bottomed 35-mm dishes and incubated for 10 min with 5 μ M DCF. The DCF fluorescence at an emission wavelength of 510 nm was observed at room temperature by exciting DCF at 488 nm using a video image analysis system (Aquacosmos, Hamamatsu Photonics).

Measurement of Rac Activity—Activation of Rac was measured by the method of Ren and Schwartz (35) with a slight modification. Forty-eight h after adenovirus infection, the cardiomyocytes were stimulated by Ang II and lysed in buffer containing 50 mM Tris (pH 7.2), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM

MgCl $_2$, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 4 μ g of GST-PAK-Cdc42/Rac interactive binding domain. Supernatant was incubated with glutathione-Sepharose beads for 2 h at 4 °C. The beads were washed and finally suspended in SDS sample buffer. Pulled down Rac was detected with anti-Rac1 antibody.

Measurement of Rho Activity—Rho activation was determined by the method of Maruyama *et al.* (32). Cells were stimulated by Ang II (100 nM) for 1 min, and lysed in buffer containing 50 mM Tris (pH 7.5), 0.1% Triton X-100, 10% glycerol, 150 mM NaCl, 30 mM MgCl $_2$, 1 mM dithiothreitol, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride. The supernatant of cell lysates was incubated with 12 μ g of GST-Rho-binding domain and glutathione-Sepharose beads for 120 min at 4 °C. The bead was washed, and finally suspended in SDS sample buffer. Pulled down Rho was detected with anti-Rho antibody.

Measurement of G α_{13} Activity—HEK293 cells in six-well dishes were transfected with rat wild type AT1R and G α_{13} with or without respective RGS domains, using FuGENE 6 reagent. Cardiomyocytes in 60-mm dishes were infected with LacZ, p115-RGS, or GRK2-RGS at 100 MOI or with CA-G α_{13} at 30 MOI. Forty-eight h after transfection, activation of G α_{13} was measured by the method of Yamaguchi *et al.* (36). After Ang II stimulation, the cells were harvested with 500 μ l of ice-cold lysis buffer containing 20 mM Hepes (pH 8.0), 2 mM MgCl $_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 10 μ g of GST-TPR. The cell lysates were then centrifuged for 5 min at 12,000 \times g, and the supernatants were incubated with glutathione-Sepharose bead for 120 min at 4 °C. The bead was washed and finally suspended in SDS sample buffer. Pulled down G α_{13} was detected with anti-G α_{13} antibody.

Intracellular Ca $^{2+}$ Measurement—The intracellular Ca $^{2+}$ concentration ([Ca $^{2+}$] $_i$) of cardiomyocytes was determined as described previously (21, 33). Briefly, the cells (1×10^6) were plated on gelatin-coated glass-bottomed 35-mm dishes and were loaded with 2.5 μ M Fura-2/AM in the cultured medium at 37 °C for 30 min. The cells were washed with Tyrode solution containing 118 mM NaCl, 5.4 mM KCl, 2 mM CaCl $_2$, 2 mM MgCl $_2$, 10 mM Hepes (pH 7.4), 0.33 mM NaH $_2$ PO $_4$, 10 mM glucose, and 30 mM taurine. Fluorescence images of green fluorescent protein-positive cells were recorded and analyzed with a video image analysis system (Aquacosmos, Hamamatsu Photonics). All of the ratio data were calculated for determination of [Ca $^{2+}$] $_i$ with *in vivo* calibration method.

Statistical Analysis—The results are shown as the means \pm S.E. The mean values were compared with control by Student's *t* test (for two groups) or one-way analysis of variance followed by Dunnett's test (for three or more groups).

RESULTS

Ang II-induced MAPK Activation—To delineate the signal transduction cascade leading to MAPK activation by Ang II stimulation, the concentration dependence and time course of MAPK activation were determined. Stimulation with Ang II resulted in a dose-dependent activation of ERK, JNK, and p38 MAPK (Fig. 1A). The EC $_{50}$ values of Ang II to activate ERK, JNK, or p38 MAPK were different between three MAPKs. ERK, JNK, and p38 MAPK activation peaked at 5, 20, and 20 min, respectively (Fig. 1B). These results demonstrate that Ang II stimulation activated three MAPKs in cardiac myocytes.

ROS Mediate Ang II-induced JNK and p38 MAPK Activation—We examined first which MAPK requires ROS for their activation. One of ROS producing systems is NADPH oxidase complex, which is activated by receptor stimulation. Thus, we examined the effects of chemical antioxidants, such as *N*-acetyl-L-cysteine (a radical scavenger), DPI (an NADPH oxidase inhibitor), and catalase (an H $_2$ O $_2$ -degrading enzyme), on MAPK activation. Ang II-induced ERK activation was not affected by these antioxidants (Fig. 1C). However, Ang II-induced activation of p38 MAPK and JNK was inhibited by all three reagents (Fig. 1, D and E). We have also found that the expression of Prx II (a radical scavenging enzyme) significantly inhibited Ang II-induced JNK activation but not ERK activation (data not shown). These results indicate that JNK and p38 MAPK activation are sensitive to ROS and suggest that ROS produced by NADPH oxidase is necessary for Ang II-induced JNK and p38 MAPK activation.

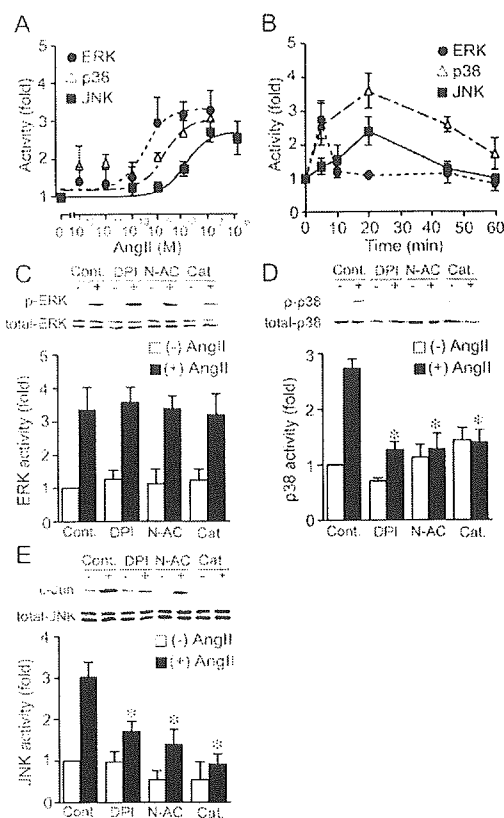


FIG. 1. ROS-dependent activation of JNK and p38 MAPK by ATR stimulation. *A*, dose-dependent activation of JNK, ERK, or p38 MAPK by Ang II stimulation. JNK, ERK, or p38 MAPK activities were measured at 20, 5, and 10 min after Ang II stimulation with the indicated concentrations. *B*, time course of JNK, ERK, or p38 MAPK activation. Ang II (100 nM) stimulation activated JNK and increased the phosphorylation of p38 MAPK, which peaked at 20 min. Ang II (10 nM) increased the phosphorylation of ERK, which peaked at 5 min. *C–E*, effects of DPI, *N*-acetyl-L-cysteine (*N*-AC) or catalase (*Cat.*) on Ang II-induced ERK (*C*), p38 MAPK (*D*), JNK (*E*) activation. Before the addition of Ang II, the cells were treated with DPI (5 μ M), *N*-acetyl-L-cysteine (300 μ M), or catalase (100 units/ml) for 20, 30, or 30 min, respectively. The results are shown as the means \pm S.E. from three to five experiments. *, $p < 0.05$ versus Ang II stimulation of control (*Cont.*) cells.

Selective Inhibition of $G_{\alpha_{12/13}}$ by the Expression of p115-RGS—To investigate which G protein is involved in Ang II-induced MAPK activation, a variety of adenoviruses that specifically inhibit G protein-mediated signaling were produced. Among them, p115-RGS or GRK2-RGS was used to specifically inhibit $G_{\alpha_{12/13}}$ or G_{α_q} , respectively. To delineate the selectivity of GRK2-RGS and p115-RGS, two RGS domains were expressed, and Ang II-stimulated increase in $[Ca^{2+}]_i$ was determined in the absence of extracellular Ca^{2+} . Ang II stimulation increased $[Ca^{2+}]_i$ (Fig. 2). Because $[Ca^{2+}]_i$ was measured in the absence of extracellular Ca^{2+} , the increase in $[Ca^{2+}]_i$ represents the activation of G_{α_q} -phospholipase C pathway. GRK2-RGS completely blocked the increase in $[Ca^{2+}]_i$, but p115-RGS did not (Fig. 2, *A* and *B*). GRK2-RGS did not affect the caffeine-induced increase in $[Ca^{2+}]_i$. Because caffeine promotes Ca^{2+} release from intracellular Ca^{2+} pool, the effect of GRK2-RGS is specific for the receptor-stimulated increase in $[Ca^{2+}]_i$. These results suggest that GRK2-RGS but not p115-RGS blocks ATR-mediated phospholipase C activation through G_{α_q} . We have also examined the specificity of p115-RGS. Because signaling molecule downstream of $G_{\alpha_{12/13}}$ such as $[Ca^{2+}]_i$ and cAMP has not been firmly established, we directly measured the activation of $G_{\alpha_{12/13}}$ by Ang II stimulation. It has been reported that $G_{\alpha_{12/13}}$ activation can be detected by selective pull-down of

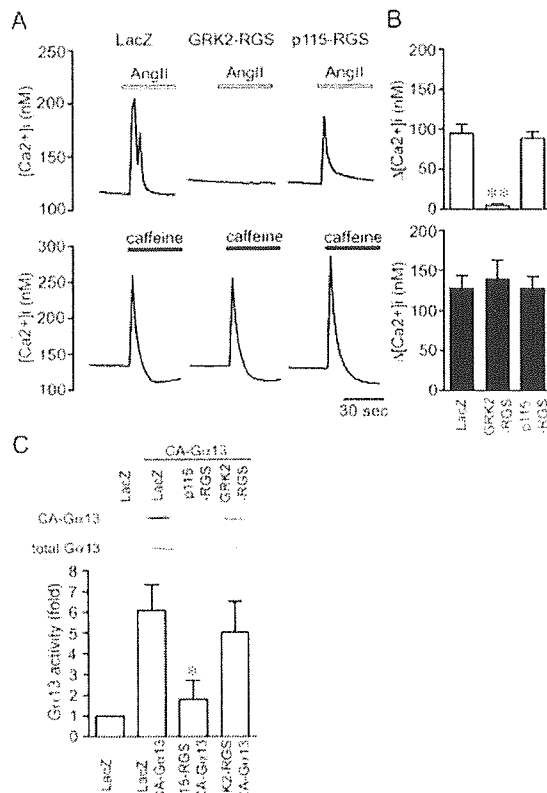


FIG. 2. Selectivity of GRK2-RGS and p115-RGS in neonatal cardiac myocytes as determined by Ang II-induced Ca^{2+} release. *A*, effects of GRK2-RGS and p115-RGS on Ang II-stimulated increases in $[Ca^{2+}]_i$. The cells were infected by adenovirus encoding LacZ (100 MOI), p115-RGS (100 MOI), or GRK2-RGS (300 MOI). Forty-eight h after infection, the cells were stimulated with 100 nM Ang II (*upper panel*) or 10 mM caffeine (*lower panel*) in Ca^{2+} -free EGTA-containing Tyrode solution. *B*, Ang II- or caffeine-induced increases in $[Ca^{2+}]_i$ were calculated and plotted. $[Ca^{2+}]_i$ was monitored from 9–22 cells, and the average trace of $[Ca^{2+}]_i$ was calculated and plotted. The experiment was repeated three times. **, $p < 0.01$ versus Ang II stimulation of LacZ-expressing cells. *C*, selectivity of p115-RGS in neonatal cardiac myocytes was determined by pull-down assay. Pull-down assay was performed by pull-down of active form of $G_{\alpha_{13}}$ using GST-TPR. The cells were co-infected with adenoviruses encoding LacZ and CA- $G_{\alpha_{13}}$, p115-RGS and CA- $G_{\alpha_{13}}$, or GRK2-RGS and CA- $G_{\alpha_{13}}$ at 50 MOI, respectively. The cell lysates were incubated with GST-TPR, and the bound $G_{\alpha_{13}}$ was detected by immunoblotting with anti- $G_{\alpha_{13}}$ antibody. *, $p < 0.05$ versus LacZ and CA- $G_{\alpha_{13}}$ -expressing cells. The experiment was repeated three times.

activated $G_{\alpha_{12/13}}$ using the TPR domain of protein phosphatase type 5 (36). Then we examined whether p115-RGS selectively inhibits Ang II receptor-stimulated $G_{\alpha_{12/13}}$ activation by pull-down assay, using CA- $G_{\alpha_{13}}$ as a positive control. Expression of CA- $G_{\alpha_{13}}$ increased the amount of CA- $G_{\alpha_{13}}$ pulled down by GST-TPR (Fig. 2*C*). The amount of pulled down $G_{\alpha_{13}}$ was completely abolished by p115-RGS, but not by GRK2-RGS. Because p115-RGS encodes RGS domain specific for $G_{\alpha_{12/13}}$ and the RGS domain can bind G_{α} in active conformation, the mechanism of this inhibition by p115-RGS is competition between p115-RGS and GST-TPR for the binding of CA- $G_{\alpha_{13}}$. By considering that p115-RGS did not affect the Ang II-induced increase in $[Ca^{2+}]_i$, it is concluded that p115-RGS specifically binds the active form of $G_{\alpha_{13}}$.

Activation of $G_{12/13}$ by ATR Stimulation—In AT1R- and $G_{\alpha_{13}}$ -expressing HEK293 cells, AT1R stimulation rapidly activated $G_{\alpha_{13}}$ (Fig. 3*A*). The activation reached a maximum at 3 min and sustained more than 2-fold for about 10 min. The Ang II-induced $G_{\alpha_{13}}$ activation was completely inhibited by p115-RGS, but not by GRK2-RGS (Fig. 3*B*). Because the RGS domain

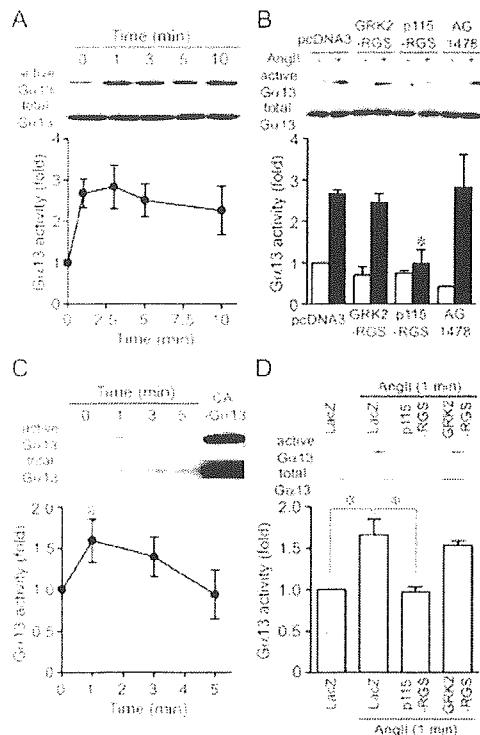


FIG. 3. Activation of $G\alpha_{13}$ by AT1R stimulation in HEK293 cells and cardiac myocytes. Selectivity of p115-RGS in HEK293 cells was determined by pull-down assay. *A*, time-dependent activation of $G\alpha_{13}$ by AT1R stimulation in wild type AT1R- and $G\alpha_{13}$ -expressing HEK293 cells. The cell lysates were incubated with GST-TPR, and bound $G\alpha_{13}$ was detected by immunoblotting with anti- $G\alpha_{13}$ antibody. *B*, effects of p115-RGS, GRK2-RGS or AG1478 on Ang II-stimulated activation of $G\alpha_{13}$. HEK293 cells were transfected with p115-RGS or GRK2-RGS, with AT1R and $G\alpha_{13}$. The cells were treated with AG1478 (500 nM) for 20 min before the addition of Ang II (100 nM, 3 min). The experiments were repeated five times. *, $p < 0.05$ versus Ang II stimulation of pcDNA3-transfected cells. *C*, time-dependent activation of $G\alpha_{13}$ by ATR stimulation in neonatal cardiac myocytes. The cardiac myocytes were stimulated with Ang II (100 nM) for the indicated times. For positive control, the cells were infected with CA- $G\alpha_{13}$ (30 MOI). *D*, effect of p115-RGS or GRK2-RGS on Ang II-induced activation of $G\alpha_{13}$ in cardiac myocytes. The cells were stimulated with Ang II (100 nM) for 1 min. The experiments were repeated five to six times. *, $p < 0.05$, as statistically analyzed by Student's *t* test.

of p115-RGS has the ability to accelerate GTPase activity of $G\alpha_{12/13}$, the mechanism of this inhibition by p115-RGS is the inactivation of GTP-bound $G\alpha_{13}$. Fig. 3*B* also shows that AG1478 treatment did not inhibit Ang II-stimulated $G\alpha_{13}$ activation (Fig. 3*B*). This result suggests that the EGF receptor is not involved in AT1R-mediated $G\alpha_{13}$ activation. Furthermore, stimulation of endogenous ATR also activated $G\alpha_{13}$ in rat cardiac myocytes (Fig. 3*C*). The activation reached a maximum at 1 min and gradually decreased to the basal level within 5 min. This Ang II-induced $G\alpha_{13}$ activation was completely inhibited by p115-RGS but not by GRK2-RGS (Fig. 3*D*). These results indicate that stimulation of AT1R activates not only G_q but also G_{13} and that p115-RGS selectively inhibits Ang II-induced $G\alpha_{13}$ activation.

$G_{12/13}$ Mediates Ang II-induced JNK and p38 MAPK Activation—We examined whether $G\alpha_{12/13}$ is involved in Ang II-induced MAPK activation. Ang II-induced ERK activation was not affected by p115-RGS, but the activation of JNK and p38 MAPK was significantly inhibited by p115-RGS (Fig. 4, A–C). We also used PTX to block ATR- G_i coupling. PTX treatment and expression of GRK2-RGS did not affect Ang II-induced JNK activation (Fig. 4*D*). We also confirmed that Ang II-induced p38 MAPK activation was insensitive to PTX or GRK2-

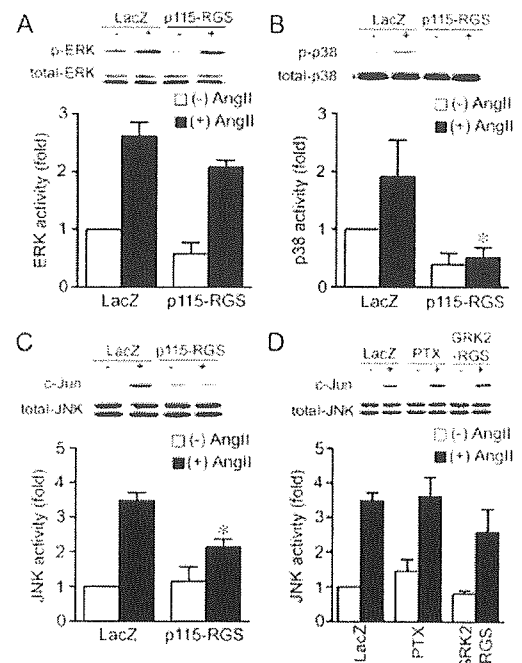


FIG. 4. Effects of p115-RGS on Ang II-stimulated MAPK activation. Cardiac myocytes were infected with LacZ- or p115-RGS-expressing adenoviruses. The cells were stimulated with Ang II (100 nM) for 20 min (JNK and p38 MAPK) or with 10 nM for 5 min (ERK), and ERK phosphorylation (*A*), p38 MAPK phosphorylation (*B*), or JNK activity (*C*) were determined. *D*, cells were infected by adenovirus encoding LacZ (100 MOI) or GRK2-RGS (300 MOI). Some portion of cells was treated with PTX (100 ng/ml) for 24 h before the addition of Ang II. The fold activation was calculated by the values of untreated cells infected with LacZ set as 1. *, $p < 0.05$ versus Ang II stimulation of LacZ-expressing cells. The experiments were repeated three to ten times.

RGS ($n = 2$; data not shown). These results suggest that the activation of JNK and p38 MAPK is mainly mediated by $G\alpha_{12/13}$ but not G_q and G_i . These results indicate that ATR couples with $G_{12/13}$, and $G\alpha_{12/13}$ activates signal transduction cascade, leading to JNK and p38 MAPK activation.

ROS-dependent Activation of JNK and p38 MAPK Induced by $G\alpha_{12/13}$ Activation—We next determined the relationship between $G\alpha_{12/13}$ and ROS in Ang II-induced JNK and p38 MAPK activation. Expression of CA- G_q , CA- G_{12} , or CA- G_{13} resulted in activation of all three MAPKs (Fig. 5). This result indicates that activated G_q , G_{12} , or G_{13} can induce activation of MAPKs in neonatal cardiac myocytes. Furthermore, G_{12} - or G_{13} -induced activation of p38 MAPK (Fig. 5*B*) and JNK (Fig. 5*C*), but not ERK (Fig. 5*A*), was sensitive to DPI. In contrast, G_q -induced JNK and p38 MAPK activation was not affected by DPI. These results are consistent with the fact that activation of $G\alpha_{12/13}$ by ATR stimulation activates JNK and p38 MAPK through ROS production.

Rac-dependent ROS Production by ATR Stimulation—We examined whether ATR stimulation actually produces ROS in rat neonatal cardiomyocytes. Fig. 6*A* shows that exposure of Ang II increased intracellular concentration of ROS. The concentration of ROS by Ang II stimulation was compared with the amount of fluorescence generated by exogenously added H_2O_2 . Ang II stimulation increased ROS to about 4 μM , and p115-RGS and PrxII inhibited ROS production (Fig. 6*B*). However, the Ang II-induced ROS production was not affected by GRK2-RGS. These results indicate that $G\alpha_{12/13}$ but not G_q mediates Ang II-induced ROS production. Furthermore, expression of DN-Rac or treatment with DPI inhibited Ang II-induced ROS production, suggesting that NADPH oxidase mediates Ang II-induced ROS production.

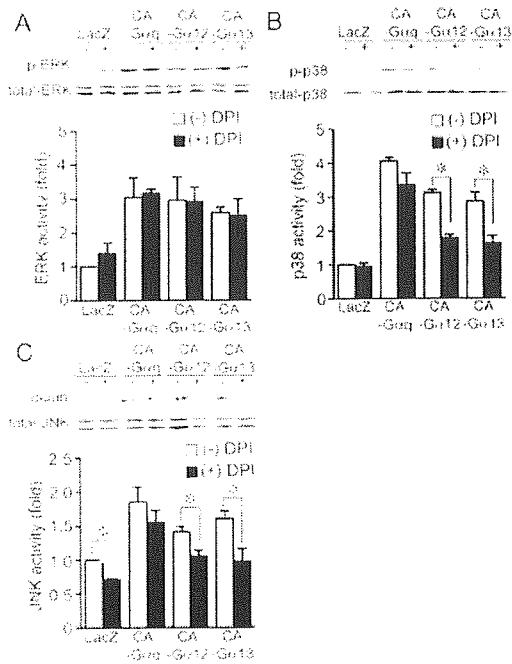


FIG. 5. ROS-dependent JNK and p38 MAPK activation or ROS-independent ERK activation following the expression of CA- $G\alpha_6$, CA- $G\alpha_{12}$, or CA- $G\alpha_{13}$. The cells were infected by adenovirus encoding LacZ (100 MOI), CA- $G\alpha_6$ (100 MOI), CA- $G\alpha_{12}$ (100 MOI), or CA- $G\alpha_{13}$ (30 MOI). Forty-eight h after infection, the cells were treated with DPI (5 μ M) for 30 min, and then ERK (A), p38 MAPK phosphorylation (B), and JNK activity (C) were determined. *, $p < 0.05$ versus MAPK activation without DPI of LacZ-, CA- $G\alpha_{12}$ -, or $G\alpha_{13}$ -expressing cells. The experiments were repeated three times.

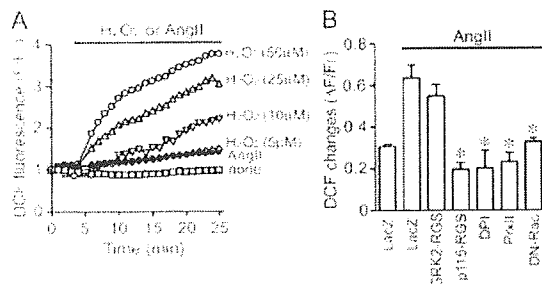


FIG. 6. Inhibition of Ang II-induced ROS production by p115-RGS and DN-Rac. A, average changes in H_2O_2 - or Ang II-induced F/F_0 of DCF from time course experiments. The cells were stimulated by the indicated concentration of H_2O_2 or by Ang II (100 nM). The traces are shown as averages from 22–36 cells. B, the increase in Ang II-induced fluorescence of DCF was calculated by the value of maximal fluorescence intensity during 25 min of stimulation and the initial value of fluorescence, F_0 . The cells were treated with DPI (5 μ M) for 20 min prior to Ang II stimulation. Ang II-stimulated DCF fluorescence was also determined in cells expressing GRK2-RGS, p115-RGS, PrxII, or DN-Rac. *, $p < 0.05$ versus Ang II stimulation of LacZ-expressing cells. The experiments were repeated three times.

$G\alpha_{12/13}$ Activates Rac Leading to JNK and p38 MAPK Activation—We examined the involvement of Rac in Ang II-induced MAPK activation. Expression of DN-Rac inhibited Ang II-induced activation of JNK and p38 MAPK, but not ERK (Fig. 7, A–C). Because DN-Rac inhibited Ang II-induced ROS production (Fig. 6B), Rac may mediate Ang II-induced JNK and p38 MAPK activation through ROS production. Rac activation can be determined by selective pull-down assay using the Cdc42/Rac interactive binding domain of PAK that is one of the Rac effectors. Rac activation was detected by angiotensin II stimulation, which peaked at 1 min and quickly returned to the basal state (data not shown). As expected, the expression of DN-Rac

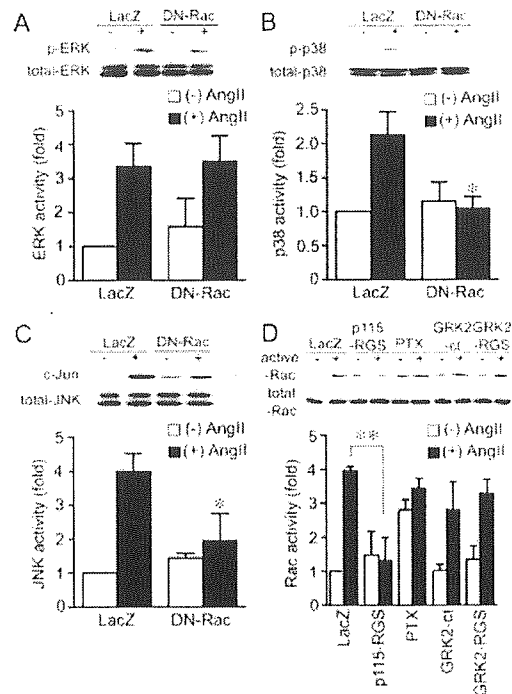


FIG. 7. Effects of DN-Rac on Ang II-stimulated MAPK activation. Cardiac myocytes were infected with adenovirus encoding LacZ or DN-Rac. A–C, cells were stimulated with Ang II (100 nM) for 20 min (JNK and p38 MAPK) or with 10 nM for 5 min (for ERK). The activation of ERK (A), p38 MAPK (B), and JNK (C) were then determined. *, $p < 0.05$ versus Ang II stimulation of LacZ-expressing cells. D, cardiac myocytes were infected with p115-RGS or GRK2-ct at 100 MOI or were infected with GRK2-RGS at 300 MOI. Some portion of cells were treated with PTX (100 ng/ml) for 24 h before the addition of Ang II. Rac activation was determined by pull-down assay. The fold activation was calculated by the values of untreated cells infected with LacZ set as 1. *, $p < 0.01$ versus Ang II stimulation of LacZ-expressing cells. The experiments were repeated three to four times.

completely inhibited Ang II-induced activation of JNK and p38 MAPK but not ERK (Fig. 7, A–C). Rac activation was inhibited by p115-RGS, but not by PTX, GRK2-ct (a $G\beta\gamma$ -sequestering polypeptide), and GRK2-RGS (Fig. 7D). The basal Rac activity of PTX-treated cells was increased for unknown reasons. Similar high basal Rac binding activity with PTX treatment has been reported by another group (37). These results suggest that $G_{12/13}$ mediate Ang II-induced Rac activation.

Rho Mediates Ang II-induced Rac Activation—Because p115-RGS inhibited JNK, p38 MAPK, and Rac activation, we speculated that $G\alpha_{12/13}$ may activate JNK, p38 MAPK, and Rac through Rho activation. Rho is specifically inactivated by C3 toxin that ADP-ribosylates Asn at position 41 of Rho. The expression of C3 toxin inhibited Ang II-stimulated JNK and Rac activation (Fig. 8, A and B). These results indicate that Rho mediates Rac and JNK activation by ATR stimulation. Because Rho regulates various kinases including ROCK, we next examined whether ROCK mediates Ang II-induced Rac activation (38). A ROCK inhibitor, Y27632, inhibited Rac activation (Fig. 8C). These results suggest that Ang II-induced Rac activation is mediated by Rho and consequent activation of ROCK. Furthermore, stimulation of ATR with Ang II activated Rho by 3-fold, which was completely inhibited by p115-RGS, but not GRK2-RGS and DN-Rac (Fig. 8D). Because ROS production is inhibited by DN-Rac, Ang II-induced Rho/ROCK activation could participate in Rac-dependent ROS production. These results also suggest that Rac activation is downstream of Rho activation.

ATR Subtype and Role of EGF Receptor Transactivation for Ang II-induced JNK Activation—Ang II-induced JNK activa-