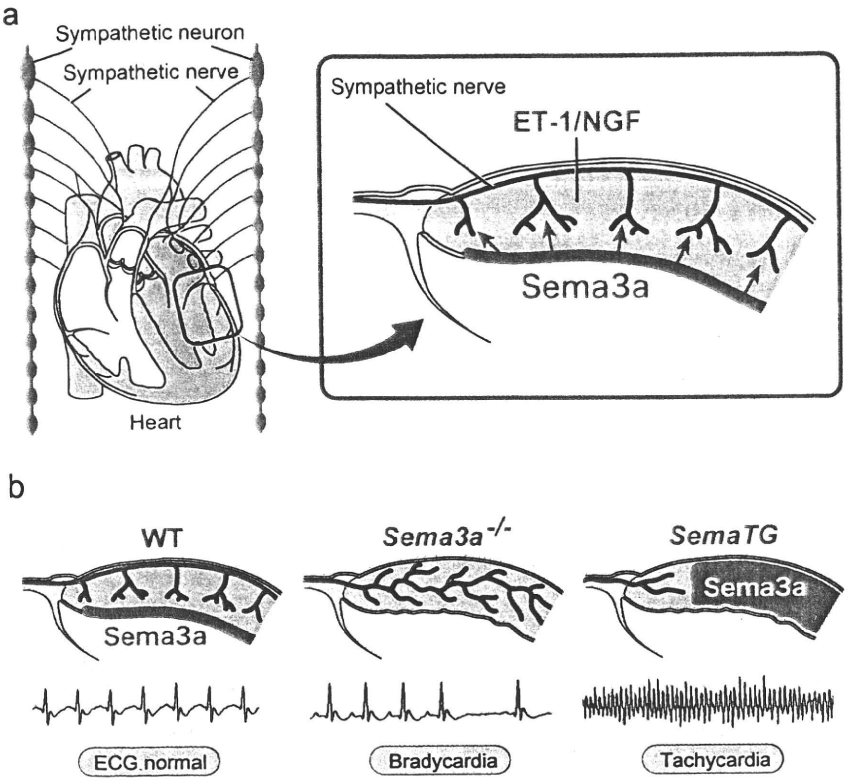

Fig. (4). Regulation of cardiac innervation patterning. (a) Cardiac sympathetic innervation shows an epicardial-to-endocardial transmural gradient. This patterning is established by the balance between ET-1/NGF and Sema3a expression in the heart. Note that NGF is expressed abundantly in the working myocardium, whereas Sema3a is expressed specifically in the subendocardium. (b) Appropriate Sema3a-mediated sympathetic innervation patterning is critical for the maintenance of an arrhythmia-free heart. *Sema3a*$^{-/-}$ mice exhibit sinus bradycardia, and *SemaTG* mice are highly susceptible to ventricular tachyarrhythmias.

tion or structural defects. Given that catecholamine augments systolic function, it is surprising that the *SemaTG* mice showed normal cardiac function. However, patients who underwent heart transplantation and had denervated hearts did not show heart failure, while approximately 10% of the patients developed SCD [91]. These results suggest that the regulation of cardiac nerves should be a new paradigm for the management of SCD.

CONCLUSIONS

Cardiac nerves are highly plastic, and innervation patterning is strictly controlled by the balance between NGF and Sema3a synthesized in the heart (Fig. **4a**). ET-1 regulates NGF expression in cardiomyocytes, and the ET-1/NGF pathway modulates nerve sprouting and plays critical roles in sympathetic nerve development [20, 40]. NGF is also important in sensory nerve development, and NGF downregulation may result in sensory neuropathy in diabetic hearts [21]. On the other hand, Sema3a inhibits neural growth and establishes appropriate innervation patterning in the heart. The disruption of sympathetic innervation patterning may lead to SCD, not only in diseased hearts but also in developing hearts (Fig. **4b**) [22]. Knowledge of the mechanisms regulating cardiac innervation patterning in hearts represents an important step towards the development of therapies for SCD.

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REFERENCES

[1] Ito, M.; Zipes, D. P. *Circulation*, **1994**, *90*, 1459-1468.
[2] Crick, S.J.; Sheppard, M. N.; Ho, S. Y.; Anderson, R. H. *J. Anat.*, **1999**, *195* (Pt 3), 341-357.
[3] Crick, S.J.; Anderson, R. H.; Ho, S.Y.; Sheppard, M.N. *J. Anat.*, **1999**, *195* (Pt 3), 359-373.
[4] Crick, S.J.; Wharton, J.; Sheppard, M.N.; Royston, D.; Yacoub, M.H.; Anderson, R.H.; Polak, J.M. *Circulation*, **1994**, *89*, 1697-1708.
[5] Cao, J.M.; Chen, L.S.; KenKnight, B.H.; Ohara, T.; Lee, M.H.; Tsai, J.; Lai, W.W.; Karagueuzian, H.S.; Wolf, P.L.; Fishbein, M.C.; Chen, P.S. *Circ. Res.*, **2000**, *86*, 816-821.
[6] Kaye, D.M.; Vaddadi, G.; Gruskin, S.L.; Du, X.J.; Esler, M.D. *Circ. Res.*, **2000**, *86*, E80-84.
[7] Cao, J.M.; Fishbein, M.C.; Han, J.B.; Lai, W.W.; Lai, A.C.; Wu, T.J.; Czer, L.; Wolf, P.L.; Denton, T.A.; Shintaku, I.P.; Chen, P.S.; Chen, L.S. *Circulation*, **2000**, *101*, 1960-1969.
[8] Chen, L.S.; Zhou, S.; Fishbein, M.C.; Chen, P.S. *J. Cardiovasc. Electrophysiol.*, **2007**, *18*, 123-127.
[9] Chen, P.S.; Chen, L.S.; Cao, J.M.; Sharifi, B.; Karagueuzian, H.S.; Fishbein, M.C. *Cardiovasc. Res.*, **2001**, *50*, 409-416.
[10] Dae, M.W.; Lee, R.J.; Ursell, P.C.; Chin, M.C.; Stillson, C.A.; Moise, N.S. *Circulation*, **1997**, *96*, 1337-1342.
[11] Qu, J.; Robinson, R.B. *J. Mol. Cell Cardiol.*, **2004**, *37*, 439-448.
[12] Faerman, I.; Faccio, E.; Milei, J.; Nunez, R.; Jadzinsky, M.; Fox, D.; Rapaport, M. *Diabetes*, **1977**, *26*, 1147-1158.
[13] Loring, J.F.; Erickson, C.A. *Dev. Biol.*, **1987**, *121*, 220-236.
[14] Francis, N.; Farinas, I.; Brennan, C.; Rivas-Plata, K.; Backus, C.; Reichardt, L.; Landis, S. *Dev. Biol.*, **1999**, *210*, 411-427.
[15] Chow, L.T.; Chow, S.S.; Anderson, R.H.; Gosling, J.A. *Br. Heart J.*, **1993**, *69*, 430-435.
[16] Hansson, M.; Kjorell, U.; Forsgren, S. *J. Mol. Cell Cardiol.*, **1998**, *30*, 2047-2057.
[17] Randall, W.C.; Szentivanyi, M.; Pace, J.B.; Wechsler, J.S.; Kaye, M.P. *Circ. Res.*, **1968**, *22*, 315-323.
[18] Schultz, H.D.; Ustinova, E.E. *Cardiovasc. Res.*, **1998**, *38*, 348-355.
[19] Hua, F.; Harrison, T.; Qin, C.; Reifsteck, A.; Ricketts, B.; Carnel, C.; Williams, C.A. *Am. J. Physiol. Heart Circ. Physiol.*, **2004**, *287*, H2728-2738.
[20] Ieda, M.; Fukuda, K.; Hisaka, Y.; Kimura, K.; Kawaguchi, H.; Fujita, J.;

Shimoda, K.; Takeshita, E.; Okano, H.; Kurihara, Y.; Kurihara, H.; Ishida, J.; Fukamizu, A.; Federoff, H.J.; Ogawa, S. *J. Clin. Invest.*, **2004**, *113*, 876-884.
[21] Ieda, M.; Kanazawa, H.; Ieda, Y.; Kimura, K.; Matsumura, K.; Tomita, Y.; Yagi, T.; Onizuka, T.; Shimoji, K.; Ogawa, S.; Makino, S.; Sano, M.; Fukuda, K. *Circulation*, **2006**, *114*, 2351-63.
[22] Ieda, M.; Kanazawa, H.; Kimura, K.; Hattori, F.; Ieda, Y.; Taniguchi, M.; Lee, J. K.; Matsumura, K.; Tomita, Y.; Miyoshi, S.; Shimoda, K.; Makino, S.; Sano, M.; Kodama, I.; Ogawa, S.; Fukuda, K. *Nat. Med.*, **2007**, *13*, 604-12.
[23] Hjalmarson, A. *Am. J. Cardiol.*, **1997**, *80*, 35J-39J.
[24] Poole-Wilson, P. A.; Swedberg, K.; Cleland, J. G.; Di Lenarda, A.; Hanrath, P.; Komajda, M.; Lubsen, J.; Lutiger, B.; Metra, M.; Remme, W. J.; Torp-Pedersen, C.; Scherhag, A.; Skene, A. *Lancet*, **2003**, *362*, 7-13.
[25] Zhou, S.; Chen, L. S.; Miyauchi, Y.; Miyauchi, M.; Kar, S.; Kangavari, S.; Fishbein, M. C.; Sharifi, B.; Chen, P. S. *Circ. Res.*, **2004**, *95*, 76-83.
[26] Snider, W. D. *Cell*, **1994**, *77*, 627-38.
[27] Lockhart, S. T.; Turrigiano, G. G.; Birren, S. J. *J. Neurosci.*, **1997**, *17*, 9573-82.
[28] Brennan, C.; Rivas-Plata, K.; Landis, S. C. *Nat. Neurosci.*, **1999**, *2*, 699-705.
[29] Heumann, R.; Korsching, S.; Scott, J.; Thoenen, H. *EMBO J.*, **1984**, *3*, 3183-9.
[30] Kanki, H.; Fukuda, K.; Okushi, K.; Ibata, I.; Toyama, Y.; Shimizu, H.; Arakawa, Y.; Ogawa, S. *Life Sci.*, **1999**, *65*, 2305-13.
[31] Clegg, D. O.; Large, T. H.; Bodary, S. C.; Reichardt, L. F. *Dev. Biol.*, **1989**, *134*, 30-7.
[32] Sakai, S.; Miyauchi, T.; Kobayashi, M.; Yamaguchi, I.; Goto, K.; Sugishita, Y. *Nature*, **1996**, *384*, 353-5.
[33] Matsumoto, Y.; Aihara, H.; Yamauchi-Kohno, R.; Reien, Y.; Ogura, T.; Yabana, H.; Masuda, Y.; Sato, T.; Komuro, I.; Nakaya, H. *Circulation*, **2002**, *106*, 613-9.
[34] Kurihara, Y.; Kurihara, H.; Oda, H.; Maemura, K.; Nagai, R.; Ishikawa, T.; Yazaki, Y. *J. Clin. Invest.*, **1995**, *96*, 293-300.
[35] Kurihara, Y.; Kurihara, H.; Suzuki, H.; Kodama, T.; Maemura, K.; Nagai, R.; Oda, H.; Kuwaki, T.; Cao, W. H.; Kamada, N.; Jishage, K.; Ouchi, Y.; Azuma, S.; Toyoda, Y.; Ishikawa, T.; Kumada, M.; Yazaki, Y. *Nature*, **1994**, *368*, 703-10.
[36] Clouthier, D. E.; Hosoda, K.; Richardson, J. A.; Williams, S. C.; Yanagisawa, H.; Kuwaki, T.; Kumada, M.; Hammer, R. E.; Yanagisawa, M. *Development*, **1998**, *125*, 813-24.
[37] Tanimoto, K.; Sugiyama, F.; Goto, Y.; Ishida, J.; Takimoto, E.; Yagami, K.; Fukamizu, A.; Murakami, K. *J. Biol. Chem.*, **1994**, *269*, 31334-7.
[38] Zhan, Y.; Kim, S.; Izumi, Y.; Izumiya, Y.; Nakao, T.; Miyazaki, H.; Iwao, H. *Arterioscler. Thromb. Vasc. Biol.*, **2003**, *23*, 795-801.
[39] Hassankhani, A.; Steinhelper, M. E.; Soonpaa, M. H.; Katz, E. B.; Taylor, D. A.; Andrade-Rozental, A.; Factor, S. M.; Steinberg, J. J.; Field, L. J.; Federoff, H. J. *Dev. Biol.*, **1995**, *169*, 309-21.
[40] Kimura, K.; Ieda, M.; Kanazawa, H.; Yagi, T.; Tsunoda, M.; Ninomiya, S.; Kurosawa, H.; Yoshimi, K.; Mochizuki, H.; Yamazaki, K.; Ogawa, S.; Fukuda, K. *Circ. Res.*, **2007**, *100*, 1755-64.
[41] Hayashi, Y.; Hussa, J. F.; Lalich, J. J. *Lab. Invest.*, **1967**, *16*, 875-81.
[42] Miyauchi, T.; Yorikane, R.; Sakai, S.; Sakurai, T.; Okada, M.; Nishikibe, M.; Yano, M.; Yamaguchi, I.; Sugishita, Y.; Goto, K. *Circ. Res.*, **1993**, *73*, 887-97.
[43] Sano, M.; Fukuda, K.; Kodama, H.; Pan, J.; Saito, M.; Matsuzaki, J.; Takahashi, T.; Makino, S.; Kato, T.; Ogawa, S. *J. Biol. Chem.*, **2000**, *275*, 29717-23.
[44] Kodama, H.; Fukuda, K.; Takahashi, E.; Tahara, S.; Tomita, Y.; Ieda, M.; Kimura, K.; Owada, K. M.; Vuori, K.; Ogawa, S. *Hypertension*, **2003**, *41*, 1372-9.
[45] Kawaguchi-Manabe, H.; Ieda, M.; Kimura, K.; Manabe, T.; Miyatake, S.; Kanazawa, H.; Kawakami, T.; Ogawa, S.; Suematsu, M.; Fukuda, K. *Life Sci.*, **2007**, *81*, 385-92.
[46] Wang, L.; Wang, D. H. *Circulation*, **2005**, *112*, 3617-23.
[47] Pan, H. L.; Chen, S. R. *Circulation*, **2004**, *110*, 1826-31.
[48] Schratzberger, P.; Walter, D. H.; Rittig, K.; Bahlmann, F. H.; Pola, R.; Curry, C.; Silver, M.; Krainin, J. G.; Weinberg, D. H.; Ropper, A. H.; Isner, J. M. *J. Clin. Invest.*, **2001**, *107*, 1083-92.
[49] Fernyhough, P.; Diemel, L. T.; Hardy, J.; Brewster, W. J.; Mohiuddin, L.; Tomlinson, D. R. *Eur. J. Neurosci.*, **1995**, *7*, 1107-10.
[50] Christianson, J. A.; Riekhof, J. T.; Wright, D. E. *Exp. Neurol.*, **2003**, *179*, 188-99.
[51] Unger, J. W.; Klitzsch, T.; Pera, S.; Reiter, R. *Exp. Neurol.*, **1998**, *153*, 23-34.
[52] Bierhaus, A.; Haslbeck, K. M.; Humpert, P. M.; Liliensiek, B.; Dehmer, T.; Morcos, M.; Sayed, A. A.; Andrassy, M.; Schiekofer, S.; Schneider, J. G.; Schulz, J. B.; Heuss, D.; Neundorfer, B.; Dierl, S.; Huber, J.; Tritschler, H.; Schmidt, A. M.; Schwaninger, M.; Haering, H. U.; Schleicher, E.; Kasper, M.; Stern, D. M.; Arnold, B.; Nawroth, P. P. *J. Clin. Invest.*, **2004**, *114*, 1741-51.
[53] Feldman, E. L.; Russell, J. W.; Sullivan, K. A.; Golovoy, D. *Curr. Opin. Neurol.*, **1999**, *12*, 553-63.
[54] Crowley, C.; Spencer, S. D.; Nishimura, M. C.; Chen, K. S.; Pitts-Meek, S.; Armanini, M. P.; Ling, L. H.; MacMahon, S. B.; Shelton, D. L.; Levinson, A. D.; Phillips, H.S. *Cell*, **1994**, *76*, 1001-11.
[55] Kuo, D. C.; Oravitz, J. J.; DeGroat, W. C. *Brain Res.*, **1984**, *321*, 111-8.
[56] Zahner, M. R.; Li, D. P.; Chen, S. R.; Pan, H. L. *J. Physiol.*, **2003**, *551*, 515-23.
[57] Bennett, D. L.; Dmietrieva, N.; Priestley, J. V.; Clary, D.; McMahon, S. B. *Neurosci. Lett.*, **1996**, *206*, 33-6.
[58] Trupp, M.; Ryden, M.; Jornvall, H.; Funakoshi, H.; Timmusk, T.; Arenas, E.; Ibanez, C. F. *J. Cell Biol.*, **1995**, *130*, 137-48.
[59] Kuruvilla, R.; Zweifel, L. S.; Glebova, N. O.; Lonze, B. E.; Valdez, G.; Ye, H.; Ginty, D. D. *Cell*, **2004**, *118*, 243-55.
[60] Emanueli, C.; Salis, M. B.; Pinna, A.; Graiani, G.; Manni, L.; Madeddu, P. *Circulation*, **2002**, *106*, 2257-62.
[61] Hellweg, R.; Hartung, H. D. *J. Neurosci. Res.*, **1990**, *26*, 258-67.
[62] Schmid, H.; Forman, L. A.; Cao, X.; Sherman, P. S.; Stevens, M. J. *Diabetes*, **1999**, *48*, 603-8.
[63] Hellweg, R.; Raivich, G.; Hartung, H. D.; Hock, C.; Kreutzberg, G. W. *Exp. Neurol.*, **1994**, *130*, 24-30.
[64] Futamatsu, H.; Suzuki, J.; Mizuno, S.; Koga, N.; Adachi, S.; Kosuge, H.; Maejima, Y.; Hirao, K.; Nakamura, T.; Isobe, M. *Circ. Res.*, **2005**, *96*, 823-30.
[65] Sakata, K.; Kumagai, H.; Osaka, M.; Onami, T.; Matsuura, T.; Imai, M.; Saruta, T. *Circulation*, **2002**, *106*, 620-5.
[66] Apfel, S. C.; Schwartz, S.; Adornato, B. T.; Freeman, R.; Biton, V.; Rendell, M.; Vinik, A.; Giuliani, M.; Stevens, J. C.; Barbano, R.; Dyck, P. J. *JAMA*, **2000**, *284*, 2215-21.
[67] Goss, J. R.; Goins, W. F.; Lacomis, D.; Mata, M.; Glorioso, J. C.; Fink, D. J. *Diabetes*, **2002**, *51*, 2227-32.
[68] Sasaki, K.; Chancellor, M. B.; Goins, W. F.; Phelan, M. W.; Glorioso, J. C.; de Groat, W. C.; Yoshimura, N. *Diabetes*, **2004**, *53*, 2723-30.
[69] Puschel, A. W.; Adams, R. H.; Betz, H. *Neuron*, **1995**, *14*, 941-8.
[70] Tanelian, D. L.; Barry, M. A.; Johnston, S. A.; Le, T.; Smith, G. M. *Nat. Med.*, **1997**, *3*, 1398-401.
[71] Kawasaki, T.; Bekku, Y.; Suto, F.; Kitsukawa, T.; Taniguchi, M.; Nagatsu, I.; Nagatsu, T.; Itoh, K.; Yagi, T.; Fujisawa, H. *Development*, **2002**, *129*, 671-80.
[72] Tago, H.; Kimura, H.; Maeda, T. *J. Histochem. Cytochem.*, **1986**, *34*, 1431-8.
[73] Kupershmidt, S.; Yang, T.; Anderson, M. E.; Wessels, A.; Niswender, K. D.; Magnuson, M. A.; Roden, D. M. *Circ. Res.*, **1999**, *84*, 146-52.
[74] Behar, O.; Golden, J. A.; Mashimo, H.; Schoen, F. J.; Fishman, M. C. *Nature*, **1996**, *383*, 525-8.
[75] Taniguchi, M.; Yuasa, S.; Fujisawa, H.; Naruse, I.; Saga, S.; Mishina, M.; Yagi, T. *Neuron*, **1997**, *19*, 519-30.
[76] Gulick, J.; Subramaniam, A.; Neumann, J.; Robbins, J. *J. Biol. Chem.*, **1991**, *266*, 9180-5.
[77] Tang, X. Q.; Tanelian, D. L.; Smith, G. M. *J. Neurosci.*, **2004**, *24*, 819-27.
[78] Kitsukawa, T.; Shimizu, M.; Sanbo, M.; Hirata, T.; Taniguchi, M.; Bekku, Y.; Yagi, T.; Fujisawa, H. *Neuron*, **1997**, *19*, 995-1005.
[79] Wright, D. E.; White, F. A.; Gerfen, R. W.; Silos-Santiago, I.; Snider, W. D. *J. Comp. Neurol.*, **1995**, *361*, 321-33.
[80] Shusterman, V.; Usiene, I.; Harrigal, C.; Lee, J. S.; Kubota, T.; Feldman, A. M.; London, B. *Am. J. Physiol. Heart Circ. Physiol.*, **2002**, *282*, H2076-83.
[81] Saba, S.; London, B.; Ganz, L. *J. Cardiovasc. Electrophysiol.*, **2003**, *14*, 191-5.
[82] Wehrens, X. H.; Lehnart, S. E.; Reiken, S. R.; Deng, S. X.; Vest, J. A.; Cervantes, D.; Coromilas, J.; Landry, D. W.; Marks, A. R. *Science*, **2004**, *304*, 292-6.
[83] Kannankeril, P. J.; Mitchell, B. M.; Goonasekera, S. A.; Chelu, M. G.; Zhang, W.; Sood, S.; Kearney, D. L.; Danila, C. I.; De Biasi, M.; Wehrens, X. H.; Pautler, R. G.; Roden, D. M.; Taffet, G. E.; Dirksen, R. T.; Anderson, M. E.; Hamilton, S. L. *Proc. Natl. Acad. Sci. USA*, **2006**, *103*, 12179-84.
[84] Kuo, H. C.; Cheng, C. F.; Clark, R. B.; Lin, J. J.; Lin, J. L.; Hoshijima, M.; Nguyen-Tran, V. T.; Gu, Y.; Ikeda, Y.; Chu, P. H.; Ross, J.; Giles, W. R.; Chien, K. R. *Cell*, **2001**, *107*, 801-13.
[85] Opthof, T.; Misier, A. R.; Coronel, R.; Vermeulen, J. T.; Verberne, H. J.; Frank, R. G.; Moulijn, A. C.; van Capelle, F. J.; Janse, M. J. *Circ. Res.*, **1991**, *68*, 1204-15.
[86] Priori, S. G.; Corr, P. B. *Am. J. Physiol.*, **1990**, *258*, H1796-805.
[87] Brunet, S.; Aimond, F.; Li, H.; Guo, W.; Eldstrom, J.; Fedida, D.; Yamada, K. A.; Nerbonne, J. M. *J. Physiol.*, **2004**, *559*, 103-20.
[88] Costantini, D. L.; Arruda, E. P.; Agarwal, P.; Kim, K. H.; Zhu, Y.; Zhu, W.; Lebel, M.; Cheng, C. W.; Park, C. Y.; Pierce, S. A.; Guerchicoff, A.; Pollevick, G. D.; Chan, T. Y.; Kabir, M. G.; Cheng, S. H.; Husain, M.; Antzelevitch, C.; Srivastava, D.; Gross, G. J.; Hui, C. C.; Backx, P. H.; Bruneau, B. G. *Cell*, **2005**, *123*, 347-58.
[89] Sosunov, E. A.; Anyukhovsky, E. P.; Gainullin, R. Z.; Plotnikov, A.; Danilo, P., Jr.; Rosen, M. R. *Cardiovasc. Res.*, **2001**, *51*, 659-69.
[90] Stramba-Badiale, M.; Lazzarotti, M.; Schwartz, P. J. *Am. J. Physiol.*, **1992**, *263*, H1514-22.
[91] Chantranuwat, C.; Blakey, J. D.; Kobashigawa, J. A.; Moriguchi, J. D.; Laks, H.; Vassilakis, M. E.; Fishbein, M. C. *J. Heart Lung Transplant.*, **2004**, *23*, 683-9.

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Tissue- and Context-Dependent Modulation of Hormonal Sensitivity of Glucocorticoid-Responsive Genes by Hexamethylene Bisacetamide-Inducible Protein 1

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Physiological and pharmacological processes mediated by glucocorticoids involve tissue- and context-specific regulation of glucocorticoid-responsive gene expression via glucocorticoid receptor (GR). However, the molecular mechanisms underlying such highly coordinated regulation of glucocorticoid actions remain to be studied. We here addressed this issue using *atp1a1* and *scnn1a*, both of which are up-regulated in response to corticosteroids in human embryonic kidney-derived 293 cells, but resistant in liver-derived HepG2 cells. Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) represses gene expression via, at least, two distinct mechanisms, *i.e.* positive transcription elongation factor b sequestration and direct interaction with GR, and is relatively high in HepG2 cells compared with 293 cells. Given this, we focused on the role of HEXIM1 in transcriptional regulation of these GR target genes. In HepG2 cells, hormone resistance of *atp1a1* and *scnn1a* was diminished by either knockdown of HEXIM1 or overexpression of GR. Such a positive effect of exogenous expression of GR was counteracted by concomitant overexpression of HEXIM1, indicating the balance between GR and HEXIM1 modulates hormonal sensitivity of these genes. In support of this, the hormone-dependent recruitment of RNA polymerase II onto *atp1a1* promoter was in parallel with that of GR. Moreover, we revealed that not positive transcription elongation factor b-suppressing activity but direct interaction with GR of HEXIM1 plays a major role in suppression of promoter recruitment of the receptor and subsequent *atp1a1* and *scnn1a* gene activation. Collectively, we may conclude that HEXIM1 may participate in tissue-selective determination of glucocorticoid sensitivity via direct interaction with GR at least in certain gene sets including *atp1a1* and *scnn1a*. (*Molecular Endocrinology* 22: 2609–2623, 2008)

GLUCOCORTICOIDS ARE secreted from the adrenal glands under the strict control of the hypothalamic-pituitary-adrenal axis and maintain homeostasis through the regulation of electrolyte balance, glucose homeostasis, lipid and protein metabolism, and modulation of the immune, cardiovascular, and central nervous system (1–3). On the other hand, glucocorticoids have been widely and successfully used in treating a number of pathological states, *e.g.* inflammation and autoimmune disorders (4). It has been demonstrated that such physiological and pharmacological processes mediated by glucocorticoids involve tissue-specific regulation of glucocorticoid-responsive gene expression (3). Moreover, glucocorticoid sensitivity of every single gene has been shown to differ among cells, tissues, and individuals, and even fluctuates not only in pathological states, but also during normal physiological processes, including development and the cell cycle (4, 5). Albeit its importance, the molecular mechanisms underlying highly coordinated tissue- and context-dependent regulation of expression of glucocorticoid-target genes remain to be studied.

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* N.S. and N.Y. contributed equally to this work, and both should be considered as first authors.

Abbreviations: AhR, Arylhydrocarbon receptor; BR, basic region; CDK9, cyclin-dependent kinase 9; ChIP, chromatin immunoprecipitation; CycT1, cyclin T1; DEX, dexamethasone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HA, hemagglutinin; HSF, heat shock factor; 3MC, 3-methylcholantrene; MOI, multiplicity of infection; PPAR, peroxisome proliferator-activated receptor; PPARRE, PPAR response element; qRT-PCR, quantitative real-time RT-PCR; P-TEFb, positive transcription elongation factor b; RNAPII, RNA polymerase II; siRNA, small interfering RNA; SDS, sodium dodecyl sulfate; snRNA, small nuclear RNA; SR, siRNA-resistant; STAT3, signal transducer and activator of transcription 3; SV, simian virus; TGZ, troglitazone; TSA, trichostatin A.

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atp1a1 is expressed in all mammalian cells, and its product Na^+, K^+-ATPase α1 plays essential roles in regulating ionic intracellular milieu, the process that is needed for the regulation of metabolism, proliferation, differentiation, and cell volume (6). In addition, in kidney, Na^+, K^+-ATPase α1 also plays a central role in the fine control of systemic electrolyte balance through hormone-regulated sodium reabsorption, in functional cooperation with amiloride-sensitive Na^+ channel, which is encoded by *scnn1a* (7–9). *atp1a1*, as well as *scnn1a*, is up-regulated in response to corticosteroids in kidney (7). On the other hand, particularly in liver, expression of either *atp1a1* or *scnn1a* is not influenced by glucocorticoids, indicating that these genes are resistant to glucocorticoids, not in kidney but in liver. Interestingly, their hormone resistance seems to be corticosteroid signal selective, because other extracellular stimuli, such as thyroid hormones (10) and low external potassium ion (11), were shown to modulate mRNA expression of *atp1a1* even in liver.

Glucocorticoids elicit their hormone actions via a signal pathway involving ubiquitously expressed glucocorticoid receptor (GR), a prototypic member of the nuclear receptor superfamily, which acts as a ligand-dependent transcription factor (12). It is generally believed that, upon binding glucocorticoids, GR translocates into the nucleus and binds the glucocorticoid response element (GRE) on the target gene promoters. Binding of liganded receptors with target DNA is followed by recruitment of mediators and coactivators to the proximity of the target DNA, resulting in RNA polymerase II (RNAPII) recruitment and activation of transcription (4, 13–19). The recent advent of DNA microarray technology has revealed that there are only modest overlaps in glucocorticoid-regulated gene sets among different cell types. In fact, considerable numbers of genes are responsive to glucocorticoids in certain tissues but resistant in others (20–23). Already several mechanisms have been postulated for tissue-specific regulation of glucocorticoid actions including different metabolisms of ligands (24), tissue-specific cofactor availability (25), and GR subtype distribution (26).

Hexamethylene bisacetamide-inducible protein 1 (HEXIM1) was originally identified as a nuclear protein, expression of which was induced when human vascular smooth muscle cells were treated with hexamethylene bisacetamide, an inhibitor of cell proliferation (27). HEXIM1 has been shown to regulate mRNA expression via, at least, two distinct mechanisms, *i.e.* positive transcription elongation factor b (P-TEFb)-dependent (28, 29) and P-TEFb-independent mechanisms (30). P-TEFb, typically composed of cyclin-dependent kinase 9 (CDK9) and its regulatory partner cyclin T1 (CycT1), phosphorylates the C-terminal domain of RNAPII, thereby stimulating transcription elongation (31–33). P-TEFb recruitment has been reported in diverse class II promoters in association with a certain class of transcription factors, including HIV-1 Tat (34), nuclear factor-κB (35), signal transducer and activator of transcription 3 (STAT3) (36), heat shock factor (HSF) (37), and arylhydrocarbon receptor (AhR) (38). HEXIM1 exerts its inhibitory effect on P-TEFb *in vivo* and *in vitro* in a 7SK small nuclear RNA (snRNA)-dependent fashion (28, 29). Upon binding with HEXIM1 and 7SK snRNA, P-TEFb looses its kinase activity, resulting in suppression of transcription elongation (39, 40). On the other hand, several reports described P-TEFb-independent mechanisms of gene regulation by HEXIM1 (30, 41–47). Among others, we demonstrated that HEXIM1 directly interacts with GR and modulates glucocorticoid-responsive gene expression (30). Moreover, we recently showed that GR, via its hinge region, interacts with central basic amino acid-rich region of HEXIM1 (48). At this moment, however, it remains unknown how genes can differentially use these distinct functions of HEXIM1: inhibitory effects on P-TEFb-dependent elongation and GR-mediated transactivation.

In the present study, we showed that mRNA expression of *atp1a1* and *scnn1a* was up-regulated by treatment with glucocorticoids in human embryonic kidney-derived 293 cells, but not in human liver cancer-derived HepG2 cells. Knockdown of endogenous HEXIM1 in HepG2 cells canceled glucocorticoid resistance of *atp1a1* and *scnn1a* mRNA expression. By creating a system that enables differential analysis of the above-mentioned distinct HEXIM1 functions, we revealed that not P-TEFb-suppressing activity but direct interaction with GR plays a major role in suppression of *atp1a1* activation by attenuating promoter recruitment of the receptor and RNAPII. We may conclude, therefore, that HEXIM1 may participate in tissue- and gene-selective determination of glucocorticoid sensitivity via direct interaction with GR, at least in a certain gene set that includes *atp1a1* and *scnn1a*.

RESULTS

Dexamethasone (DEX)-Resistance of *atp1a1* and *scnn1a* Not in 293 Cells but in HepG2 Cells

Expression of *atp1a1* and *scnn1a* is shown to be up-regulated by glucocorticoids, as well as aldosterone, in kidney and kidney-derived 293 cells. On the other hand, in liver, their mRNA expression is induced by various intra- or extracellular stimuli except for glucocorticoids (see "Introduction"). That is, *atp1a1* and *scnn1a* appear to be resistant rather selectively against glucocorticoid-GR system particularly in liver. To address the molecular mechanism of such tissue-dependent hormone resistance in gene regulation, we studied HepG2 cells as a model in comparison with 293 cells. When we analyzed their mRNA expression levels using quantitative real-time RT-PCR (qRT-PCR), 6 h treatment of 293 cells with 100 nM DEX induced *atp1a1* and *scnn1a* mRNA expression by 1.9-fold and

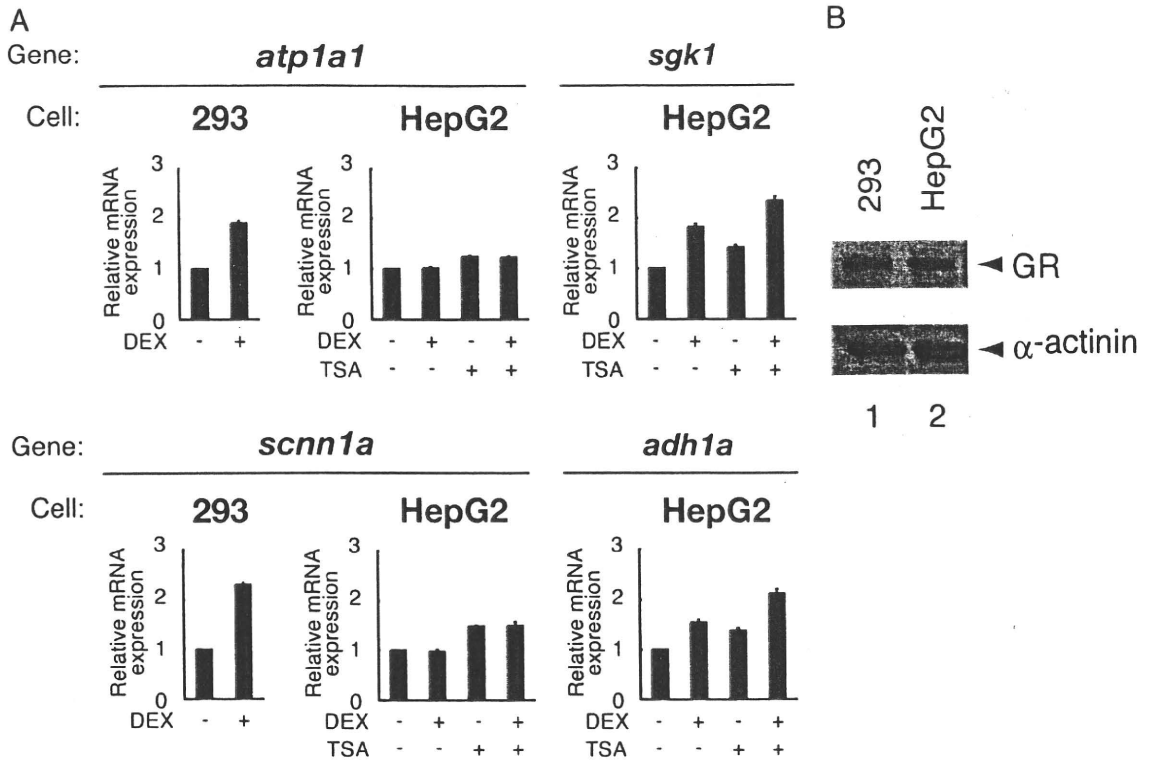

Fig. 1. DEX Resistance of *atp1a1* and *scnn1a* Not in 293 Cells but in HepG2 Cells

A, 293 cells and HepG2 cells were cultured in phenol red-free Opti-MEM I for 24 h and treated with or without 100 nM DEX for 6 h in the presence or absence of 100 nM TSA, as indicated. Total RNA was prepared and endogenous mRNA for Na^+, K^+-ATPase α1 (*atp1a1*), amiloride-sensitive Na^+ channel 1α (*scnn1a*), serum and glucocorticoid-regulated kinase 1 (*sgk1*), alcohol dehydrogenase 1A (*adh1a*), and glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was measured with qRT-PCR. Samples were normalized to *gapdh* mRNA levels, and relative expression levels to vehicle-treated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. B, Cell lysates from 293 cells and HepG2 cells were subjected to Western blot analysis using indicated antibodies.

2.3-fold, respectively (Fig. 1A). In contrast, mRNA expression of neither *atp1a1* nor *scnn1a* was induced in the presence of 100 nM DEX in HepG2 cells (Fig. 1A). Our previous DNA microarray analysis also supported this notion (data not shown). Note that GR is comparably expressed in HepG2 cells and 293 cells (Fig. 1B), and that mRNA expression of *sgk1* and *adh1a,* both of which are known to be glucocorticoid target genes as well (20–23), are induced by 1.8-fold and 1.6-fold, respectively, after DEX treatment in HepG2 cells (Fig. 1A). It is indicated, therefore, that, despite the presence of functional GR, mRNA expression of a particular set of genes, *i.e. atp1a1* and *scnn1a,* shows a cell- or tissue-specific resistance to glucocorticoids. Treatment with a histone deacetylase inhibitor trichostatin A (TSA) increased both basal and induced mRNA levels of *sgk1* and *adh1a*, but, in the case of *atp1a1* and *scnn1a*, only basal mRNA levels were increased without restoration of responsiveness to DEX (Fig. 1A). Glucocorticoid insensitivity of *atp1a1* and *scnn1a* in HepG2 cells, thus, does not appear to be related to histone acetylation-dependent mechanisms.

Cell Type Difference in Hormone-Dependent GR Recruitment onto GRE in *atp1a1* Promoter

In GR-dependent transactivation, binding of liganded receptors onto target DNA is generally believed to be an essential trigger for transcription initiation and elongation (12–15). We, therefore, tested *in vivo* occupancy of *atp1a1* promoter by endogenous GR in comparison with *sgk1* promoter using chromatin immunoprecipitation (ChIP) assay, because both promoters are known to contain GRE (Fig. 2A) (49, 50). In HepG2 cells, when *sgk1* promoter was tested, GR was recruited onto the promoter in a time- and hormone-dependent manner. In contrast, *atp1a1* promoter did not recruit GR even in the presence of DEX (Fig. 2A). Next, to examine the relationship between GR recruitment and ongoing transcription, we compared GR binding and RNAPII binding on *atp1a1* promoter after DEX treatment. RNAPII binding, as well as that of GR, was not increased after DEX treatment in HepG2 cells. In clear contrast, RNAPII binding was enhanced after DEX treatment in concert with increase of GR binding in 293 cells (Fig. 2B). These results indicate that glucocorticoid

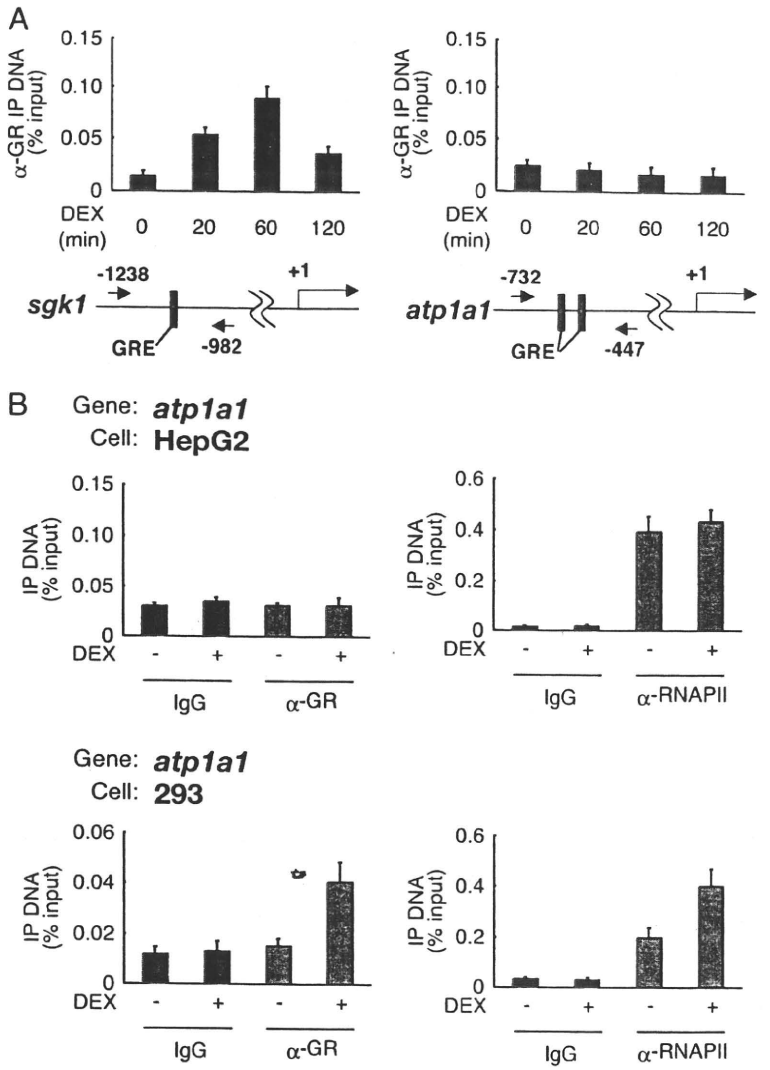

Fig. 2. Cell Type Difference in Hormone-Dependent GR Recruitment onto *atp1a1* Promoter GRE

A, HepG2 cells were cultured in phenol red-free Opti-MEM I for 24 h and treated with 1 μM DEX for the indicated time periods. ChIP assays were performed with anti-GR polyclonal antibodies, and recovered GRE-containing DNA fragments were measured with qRT-PCR. GREs in *sgk1* and *atp1a1* 5′-flanking regions are indicated as *gray boxes*. The positions of the primers are shown as *numbered arrows*. Values are expressed as percentage of immunoprecipitated DNA to input. *Error bars* represent SD values of at least three independent experiments. B, HepG2 cells and 293 cells were cultured as described in panel A and treated with 1 μM DEX for 60 min. ChIP assays were performed with the indicated antibodies and primer sets as described in *Materials and Methods*. IP, Immunoprecipitation.

resistance of *atp1a1* promoter may be due to cell type-dependent deficiency of GR recruitment despite the presence of the receptor.

Endogenous HEXIM1 Negatively Modulates Glucocorticoid-Mediated Transcriptional Activation

It was previously described that HEXIM1 mRNA is ubiquitously expressed but its expression levels are variable among human tissues (27). Protein expression levels of endogenous HEXIM1 also show a great diversity among tissues in mice; HEXIM1 expression levels appeared to be low in kidney compared with those in liver, brain, lung, spleen, and heart (Fig. 3A). These tissue-dependent differences of HEXIM1 expression levels were also observed in human tissue-derived cell lines, *i.e.* HepG2 cells abundantly express HEXIM1 compared with 293 cells (Fig. 3A). Together with the fact that certain GR target genes, *e.g. atp1a1* and *scnn1a*, are resistant to hormone treatment in HEXIM1-rich HepG2 cells, we hypothesized that HEXIM1 may participate in cell type-dependent hormone resistance at the level of transcriptional regulation of these genes.

Given this, we then addressed whether endogenous HEXIM1 contributes to glucocorticoid insensitivity of

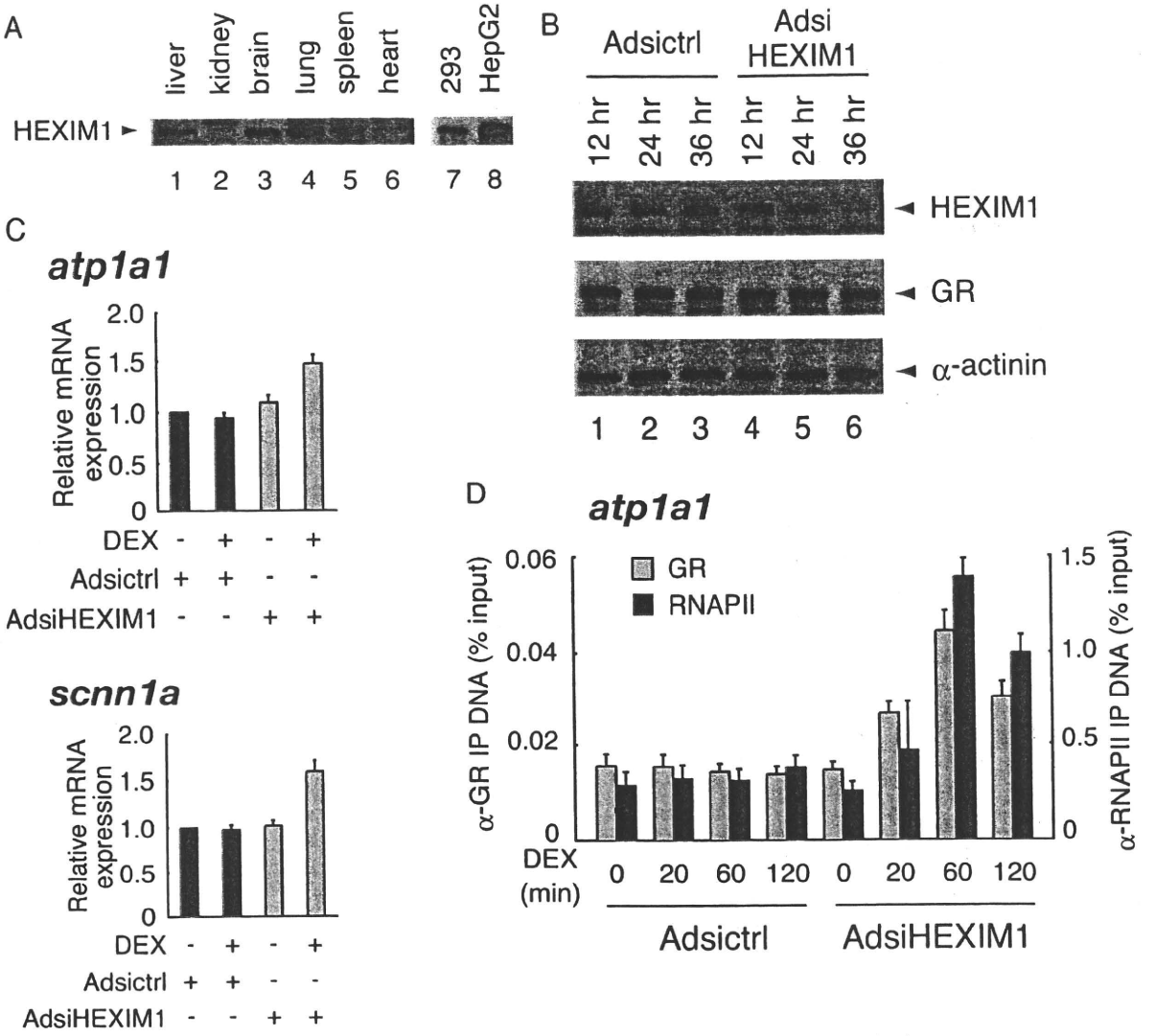

Fig. 3. Endogenous HEXIM1 Negatively Modulates Glucocorticoid-Mediated Transcriptional Activation

A, Cell lysates from various tissues from adult mice were subjected to Western blot analysis using indicated antibodies. B, HepG2 cells were infected with Adsictrl or AdsiHEXIM1 in phenol red-free Opti-MEM I at MOI of 100 for indicated time periods. Whole-cell extracts were prepared, and protein expression levels of endogenous HEXIM1, GR, and α-actinin were assessed in Western blotting. C, HepG2 cells were infected with the recombinant adenoviruses for 36 h as described in panel B and stimulated with 100 nM DEX for 6 h. Total RNA was prepared and mRNA for *atp1a1*, *scnn1a*, and *gapdh* was measured with qRT-PCR. Samples were normalized to *gapdh*, and relative expression levels to vehicle-treated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. D, HepG2 cells were infected with the recombinant adenoviruses for 36 h as described in panel B and stimulated with 1 μM DEX for indicated time periods. ChIP assays were performed with the indicated antibodies as described in *Materials and Methods*. IP, Immunoprecipitation.

atp1a1 and *scnn1a* in HepG2 cells. For that purpose, we constructed the recombinant adenoviruses expressing small interfering RNA (siRNA) against HEXIM1 named Adsi-HEXIM1, and unrelated siRNA named Adsictrl, as described in *Materials and Methods*. Figure 3B shows that infection of AdsiHEXIM1 diminished endogenous HEXIM1 protein expression down to less than 10% of the control without significant alteration of GR protein expression level. Again, mRNA expression of *atp1a1* and *scnn1a* was not significantly induced in the presence of 100 nM DEX in Adsictrl-infected HepG2 cells (Fig. 3C). Infection of AdsiHEXIM1 had little effect on basal levels of but significant effect on DEX-inducibility of *atp1a1* and *scnn1a* (Fig. 3C). Using *atp1a1* as a model, we then studied the influence of knockdown of endogenous HEXIM1 on hormone-dependent GR recruitment onto *atp1a1* promoter in ChIP analysis. As shown in Fig. 3D, GR was recruited in a time-dependent manner onto the promoter after DEX treatment in AdsiHEXIM1-infected cells. Moreover, RNAPII was also incorporated to the promoter in parallel with GR recruitment (Fig.

3D). Similar results were obtained when *scnn1a* promoter was used for ChIP assay (data not shown). These findings strongly support the notion that protein levels of endogenous HEXIM1 might determine GR recruitment onto these promoters and subsequent glucocorticoid-responsive transcription of *atp1a1* and *scnn1a* in HepG2 cells.

GR and HEXIM1 Stochastically Contribute to Hormone-Dependent Transcriptional Regulation of GR-Target Genes

To test relative contribution of GR and HEXIM1 in the expression of *atp1a1*, we overexpressed FLAG-GR alone or in combination with HEXIM1 in HepG2 cells

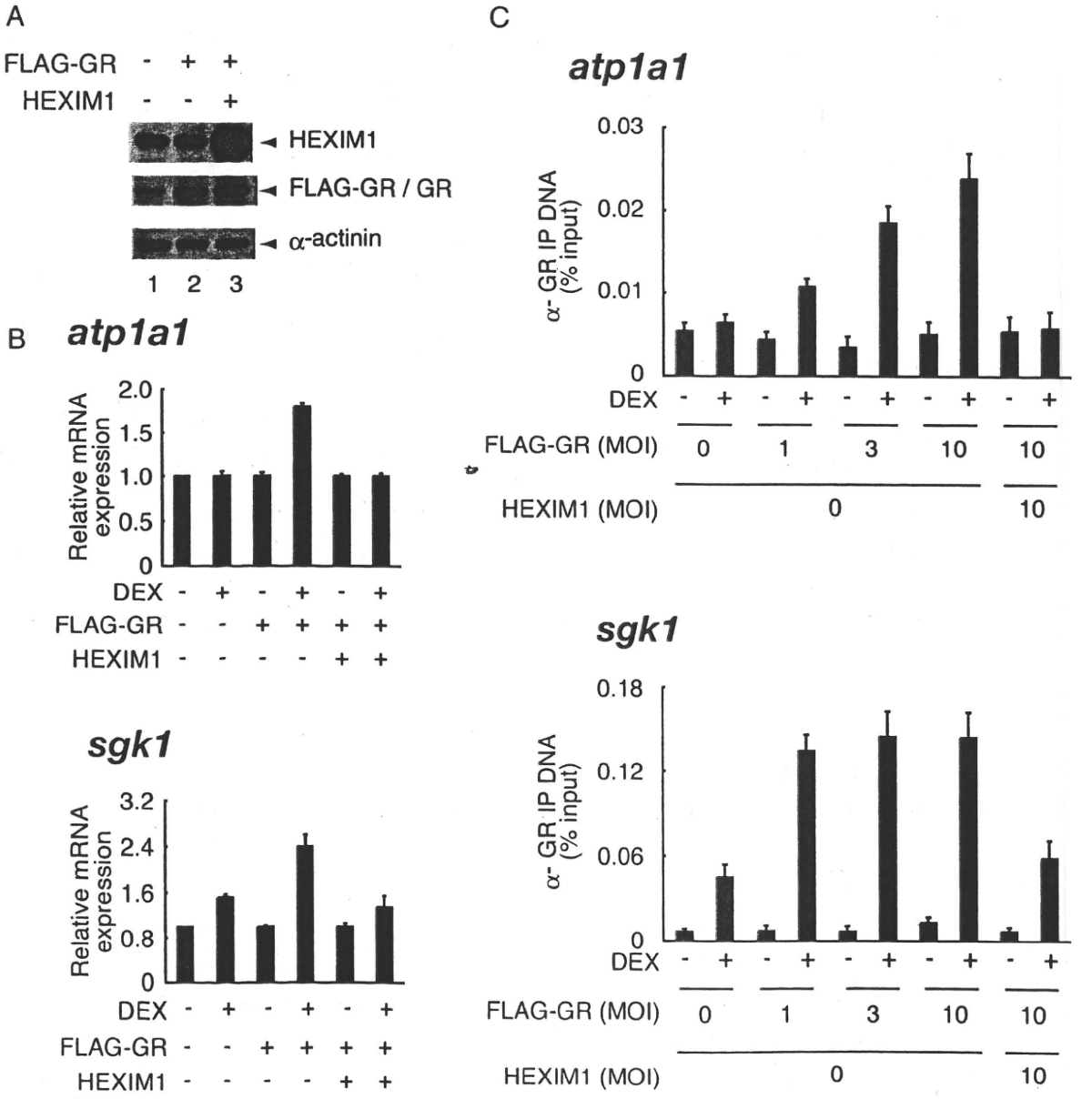

Fig. 4. GR and HEXIM1 Stochastically Contribute to Hormone-Dependent Transcriptional Regulation of GR-Target Genes

A, HepG2 cells were infected with the recombinant adenoviruses expressing FLAG-GR and HEXIM1 in phenol red-free Opti-MEM I at MOI of 5 for 24 h as indicated. Cells were lysed and subjected to Western blot analysis using the indicated antibodies. B, HepG2 cells were infected with the recombinant adenoviruses as described in panel A and treated with or without 100 nM DEX for 6 h. Total RNA was prepared, and mRNA for *atp1a1*, *sgk1*, and *gapdh* was measured with qRT-PCR. Samples were normalized to *gapdh*, and relative expression levels to vehicle-treated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. C, HepG2 cells were infected with the recombinant adenoviruses in phenol red-free Opti-MEM I for 24 h as indicated and treated with or without 1 μM DEX for 20 min. ChIP assays were performed with anti-GR antibodies as described in *Materials and Methods*. IP, Immunoprecipitation.

using recombinant adenoviruses. Western blots showed that the protein levels of expressed FLAG-GR and HEXIM1 were approximately 5-fold and 8-fold compared with those of endogenous GR and HEXIM1, respectively (Fig. 4A). In HepG2 cells, overexpression of FLAG-GR restored glucocorticoid responsiveness and resulted in DEX-dependent induction of *atp1a1* mRNA by 1.8-fold, which was again canceled by coexpression of HEXIM1 (Fig. 4B). We may propose, therefore, that high-level expression of HEXIM1 relative to GR confers tissue-specific glucocorticoid resistance of *atp1a1* in HepG2 cells. This is also the case in *scnn1a* mRNA expression (data not shown). Interestingly, mRNA expression of *sgk1* was also negatively affected by exogenous expression of HEXIM1 (Fig. 4B). Our ChIP assay revealed that GR overexpression restored hormone-dependent recruitment of GR to *atp1a1* promoter in a dose-dependent manner, which was again canceled by overexpression of HEXIM1 (Fig. 4C). In the case of *sgk1* promoter as well, overexpression of GR further increased hormone-dependent GR recruitment, which was antagonized by exogenous HEXIM1 (Fig. 4C). These findings strongly support the notion that GR and HEXIM1 stochastically contribute to hormone-dependent transcriptional regulation of both *atp1a1* and *sgk1*, and that *atp1a1* promoter is more susceptible to HEXIM1.

Knockdown of HEXIM1 Enhanced P-TEFb-Dependent Gene Expression

In addition to suppressing GR recruitment onto the target DNA, HEXIM1 is originally reported to inactivate kinase activity of P-TEFb, thereby suppressing transcription elongation (see *Introduction*). To investigate whether knockdown of HEXIM1 in our system affects P-TEFb-dependent mRNA expression as previously reported (51), we analyzed mRNA expression of several genes, expression of which has been reported to be critically regulated by P-TEFb at the step of transcription elongation.

mRNA level of *cyp1a1* was increased after stimulation with 1 h treatment of 10 nM 3-methylcholantrene (3MC) by 3.7-fold, probably via activation of AhR and subsequent recruitment of P-TEFb onto *cyp1a1* promoter (38), and AdsiHEXIM1 further enhanced this 3MC effect by 5.0-fold (Fig. 5A). IL-6-mediated expression of *junB* mRNA (14-fold), which is mediated by STAT3 (52), was also enhanced by AdsiHEXIM1 (22-fold). Finally, heat shock-mediated amplification of mRNA expression of *hspa1a* and *fos* (11-fold and 8.1-fold, respectively), which is mediated by HSF1 (53, 54), was further enhanced by

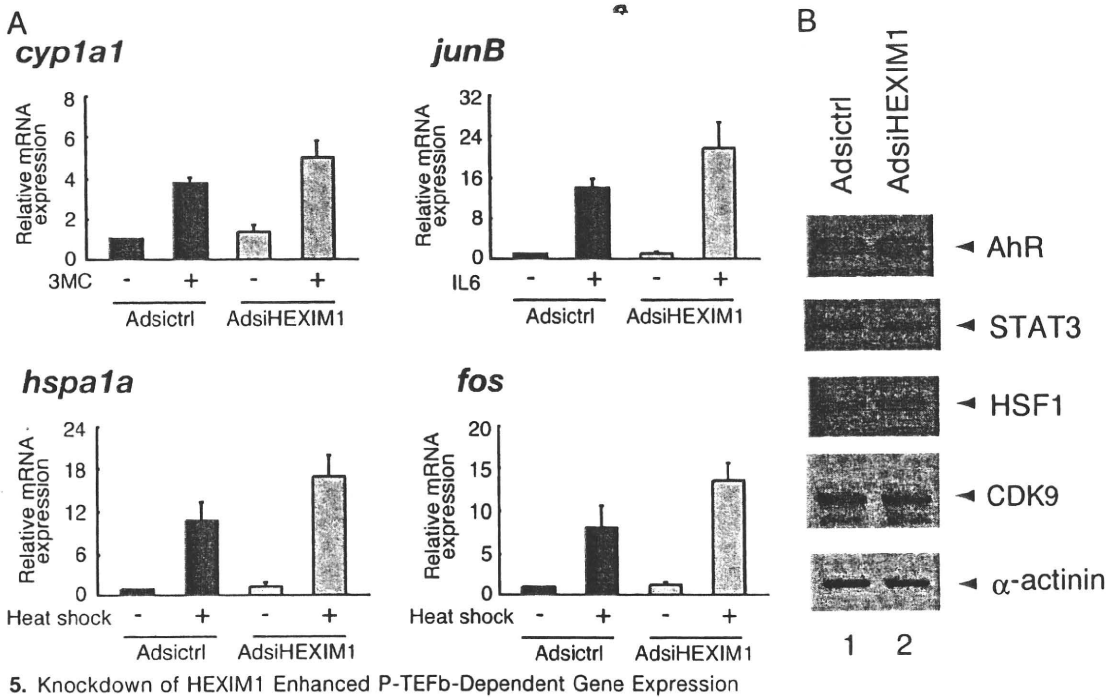

Fig. 5. Knockdown of HEXIM1 Enhanced P-TEFb-Dependent Gene Expression

A, HepG2 cells were infected with Adsictrl or AdsiHEXIM1 as described in Fig. 3B and stimulated with 10 nM 3MC, 100 ng/ml IL-6, or culture at 42 C (heat shock) for 1 h as indicated. Endogenous mRNA for cytochrome P450, family 1, subfamily A, polypeptide 1 (*cyp1a1*), JunB (*junB*), heat shock 70-kDa protein 1A (*hspa1a*), Fos (*fos*), and *gapdh* was measured with qRT-PCR. Samples were normalized to *gapdh*, and relative expression levels to the Adsictrl-infected and unstimulated samples are presented as relative mRNA expression. *Error bars* represent SD values of at least three independent experiments. B, HepG2 cells were infected with the recombinant adenoviruses and stimulated as described in panel A. Nuclear extracts were prepared, and protein expression levels of endogenous AhR, STAT3, HSF1, CDK9, and α-actinin were assessed by Western blotting.

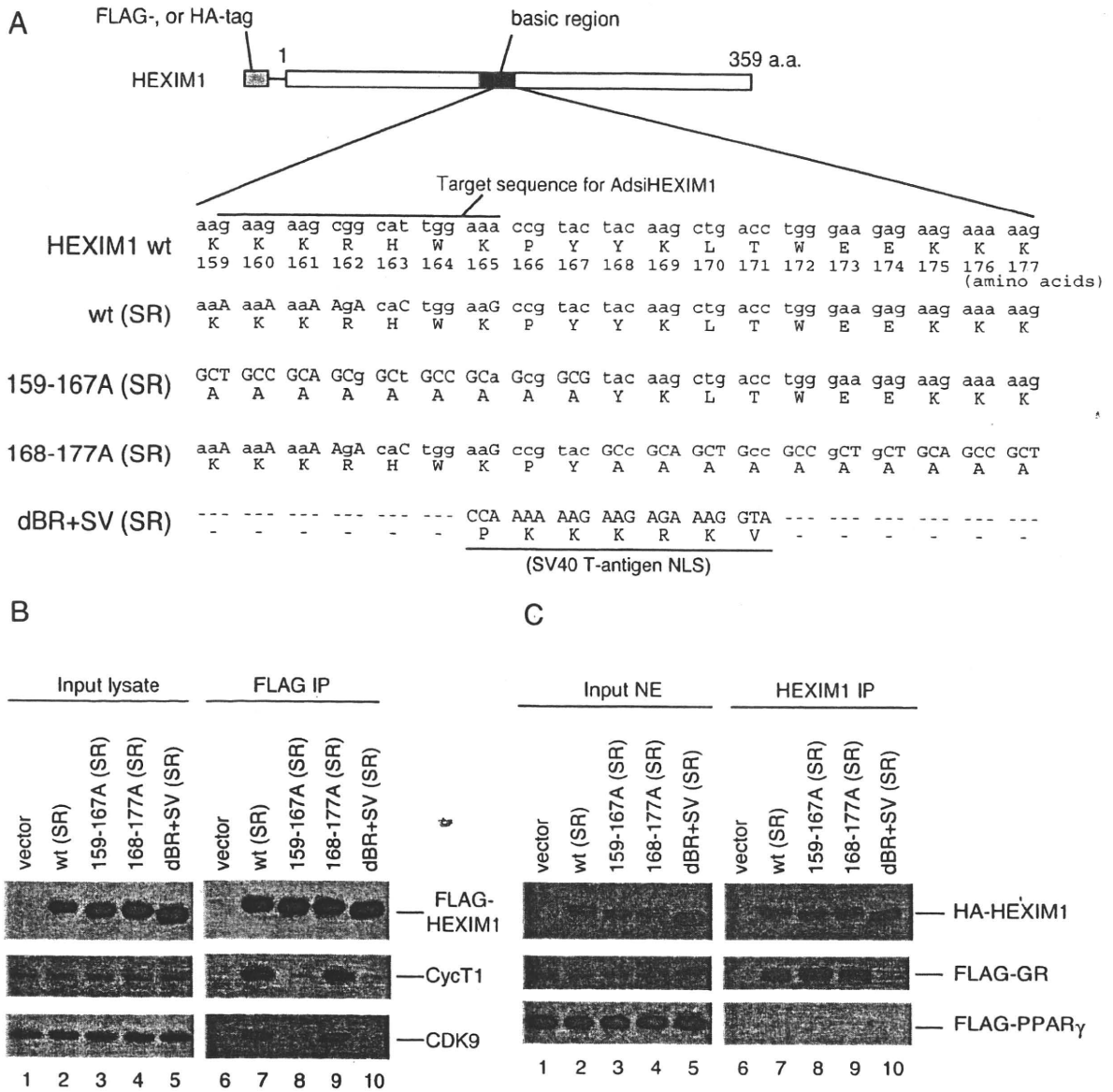

Fig. 6. P-TEFb-Binding and GR Binding Are Separable for HEXIM1

A, Schematic illustration of wild-type (wt) and mutant HEXIM1 used in this study. BR encompassing 150–177 amino acids are depicted as a *solid box*. *Numbers* depict positions of amino acids. Nucleotide and amino acid sequences in the BR are shown. Substitutions of nucleotides are shown in *uppercase letters*. wt (SR) and 168–177A (SR) have nucleotide substitutions in the target nucleotide sequence for AdsiHEXIM1 without affecting original amino acid sequence. 159–167A (SR) and dBR+SV (SR) are resistant to AdsiHEXIM1 by nature. B, HeLa cells were cotransfected with empty vector or expression plasmids for indicated FLAG-tagged mutant HEXIM1. Whole-cell lysates were prepared and subjected to FLAG-affinity purification as described in *Materials and Methods*. Western blot analysis of input lysates (lanes 1–5) and affinity-purified fractions (lanes 6–10) were performed using anti-FLAG peptide, anti-CycT1, and anti-CDK9 antibodies. C, COS7 cells were cotransfected with empty vector or expression plasmids for indicated HA-tagged mutant HEXIM1 along with either FLAG-tagged GR (*middle panel*) or FLAG-tagged PPAR γ (*lower panel*) expression plasmid. Cells were treated with 100 nM DEX (*middle panel*) or 100 nM TGZ for 2 h. Nuclear extracts were prepared and immunoprecipitated with anti-HEXIM1 antibodies. Western blot analysis of input extracts (lanes 1–5) and immunoprecipitated fractions (lanes 6–10) were performed using anti-HA peptide and anti-FLAG peptide antibodies. a.a., Amino acids; IP, immunoprecipitation; NE, nuclear extract; NLS, nuclear localization signal.

AdsiHEXIM1 (17-fold and 14-fold, respectively). Note that protein expression levels of AhR, STAT3, HSF1, and CDK9 were not significantly influenced by infection of AdsiHEXIM1 (Fig. 5B). We may conclude, therefore, that P-TEFb activity was enhanced in HEXIM1-knocked down cells and that the P-TEFb-dependent elongation process was up-regulated in certain genes.

P-TEFb Binding and GR Binding Are Separable for HEXIM1

To highlight the GR target gene-selective role of HEXIM1, we established an experimental system that enables us to clarify which function of HEXIM1 is important in regulation of GR-target gene expression, P-TEFb suppression or GR sequestration. In short, endogenous HEXIM1 was knocked down by infection of AdsiHEXIM1, and mutant HEXIM1, which lacks either P-TEFb-suppressing activity or direct interaction with GR, or both, was exogenously complemented. To obtain such a mutant HEXIM1, we focused on basic region (BR) of HEXIM1 and made alanine substitution and domain swap mutants, as schematically depicted in Fig. 6A, because we and others previously showed that BR is essential for nuclear localization, interaction with GR, and P-TEFb-inhibition (28, 30, 41, 55). Because siRNA against HEXIM1 in AdsiHEXIM1 was designed to target the region corresponding to amino acids 159–165 (Fig. 6A), the expression plasmids for siRNA-resistant wild-type (SR) and 168–177A (SR) were created with several nucleotide substitutions in HEXIM1 cDNA without affecting original amino acid sequence (Fig. 6A). In indirect immunofluorescence analysis, every mutant HEXIM1 protein was expressed in the nucleus in transfected cells (data not shown).

To verify the presence or absence of the interaction between P-TEFb and these mutant FLAG-tagged HEXIM1, we, after transfection of their expression plasmids into HeLa cells, immunoprecipitated cell lysate with anti-FLAG monoclonal antibody, and blots were probed with the antibodies against major P-TEFb subunits CycT1 and CDK9. As expected, substitution of BR to the nuclear localization signal from simian virus (SV) 40 large T antigen, resulting in dBR+SV (SR), completely abolished binding of CycT1 and CDK9 (Fig. 6B). Alanine substitution of amino acids 159–167, which was shown to disrupt the interaction with 7SK snRNA (55), diminished consecutive recruitment of CycT1 and CDK9 (Fig. 6B), as seen in dBR+SV (SR). On the other hand, alteration of amino acids 168–177 to alanines did not affect binding of CycT1 or CDK9 (Fig. 6B).

Using these HEXIM1 mutants, we also studied the physical interaction between HEXIM1 and GR. For this purpose, hemagglutinin (HA)-tagged wild-type (SR) and mutant HEXIM1 were expressed in COS7 cells along with either FLAG-tagged GR or FLAG-tagged peroxisome proliferator-activated receptor γ (PPARγ) as a control and immunoprecipitated with anti-HEXIM1 antibodies. As shown in Fig. 6C, GR bound not only wild-type (SR) but also HEXIM1 mutants with alanine substitution, but the swap mutant dBR+SV (SR) did not bind GR. These results may suggest that amino acids 159–177 of HEXIM1 are not critical for binding GR, but protein configuration of BR and its proximity is important for GR recognition. In contrast, PPARγ did not bind wild-type (SR) or any mutant HEXIM1 (Fig. 6C). To further confirm that these HEXIM1 mutants, especially 159–167A (SR), retain not P-TEFb-inhibition but GR suppression, we tested their

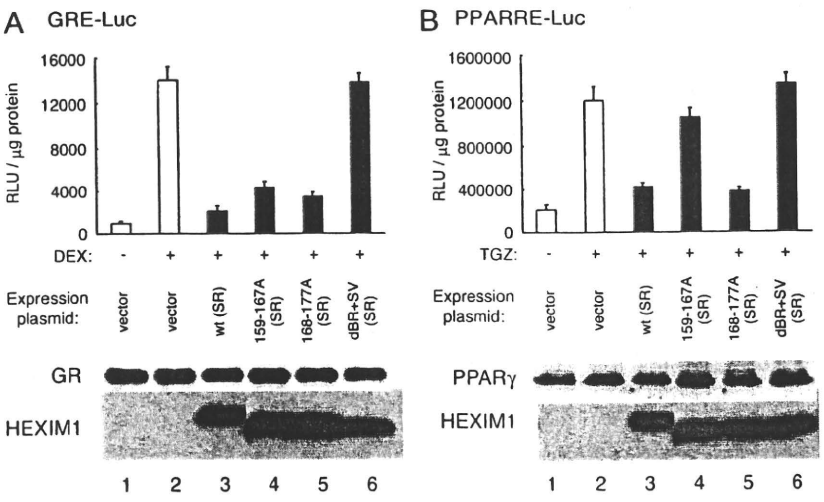

Fig. 7. Differential Functions of HEXIM1 on P-TEFb- and GR-Dependent Gene Expression

A, COS7 cells were cotransfected with empty vector or expression plasmids for the indicated HA-tagged mutant HEXIM1 along with GR expression plasmid and GRE reporter plasmid. Four hours later, media were replaced, further cultured for 20 h, and treated with vehicle or 100 nM DEX for 18 h as indicated. Cells were lysed and subjected to luciferase assay. Results are presented as relative light units (RLU) per microgram of protein in the lysates. *Error bars* represent SD values of at least three independent experiments. Protein expression levels of GR and HA-HEXIM1 were assessed in Western blotting. B, COS7 cells were cotransfected with empty vector or expression plasmids for indicated HA-tagged mutant HEXIM1 along with PPARγ expression plasmid and PPARRE reporter plasmid. Media were replaced 4 h later, further cultured for 20 h, and treated with vehicle or 100 nM TGZ for 18 h as indicated. Cells were lysed and subjected to luciferase assay. Results are presented as RLU per microgram of protein in the lysates. *Error bars* represent SD values of at least three independent experiments. Protein expression levels of PPARγ and HA-HEXIM1 were assessed by Western blotting.

functions in a GRE-luciferase reporter gene assay (Fig. 7A). PPARγ-dependent reporter gene assay served as a control (Fig. 7B), because neither wild-type (SR) nor any mutant HEXIM1 was capable of binding PPARγ (Fig. 6C). HEXIM1 dBR+SV (SR), which lacks binding activity to either P-TEFb or GR, did not significantly affect either reporter gene activity, as expected (Fig. 7, A and B, *top*). With respect to GR-driven reporter gene expression, any alanine-substituted HEXIM1 mutant suppressed ligand-dependent activation of the reporter gene as well as wild type (SR), indicating its functional interaction with GR (Fig. 7A, *top*). In clear contrast, PPARγ-mediated activation of the reporter gene was repressed solely by wild-type (SR) and 168–177A (SR) (Fig. 7B, *top*). In these experimental settings, protein expression of FLAG-tagged GR or FLAG-tagged PPARγ was not significantly affected by HEXIM1 mutants (Fig. 7, A and B, *bottom*). It is concluded, therefore, that these HEXIM1 mutants can serve an efficient tool for delineating mechanism of suppressing expression of particular genes by HEXIM1, *i.e.* P-TEFb suppression or GR binding.

P-TEFb Is Not Involved in HEXIM1-Mediated Suppression of Glucocorticoid Responsiveness

Finally, we differentially evaluated the importance of P-TEFb-suppressing and GR-binding activities of HEXIM1 in regulating glucocorticoid sensitivity of glucocorticoid-inducible mRNA expression of *atp1a1* and *scnn1a*. HeLa cells were transfected with the expression plasmids for HEXIM1 (SR) mutants, infected with AdsiHEXIM1, and treated with the cognate ligands, after which RNA was isolated for qRT-PCR analyses. In HEXIM1 knocked-down cells, mRNA expression of *cyp1a1*, which is known to be P-TEFb dependent (38), was stimulated by 10-fold in response to 6 h treatment with 10 nM 3MC (Fig. 8A). Adding back of wild-type (SR) HEXIM1 repressed induction of mRNA expression of *cyp1a1*, to 3.3-fold, suggesting that ectopically expressed HEXIM1 (SR) functionally suppressed P-TEFb activity (Fig. 8A). However, neither 159–167A (SR) nor dBR+SV (SR) repressed *cyp1a1* expression, confirming that the suppression of P-TEFb activity may be critical for the repression (Fig. 8A). In support of this, 168–177A (SR), which binds P-TEFb

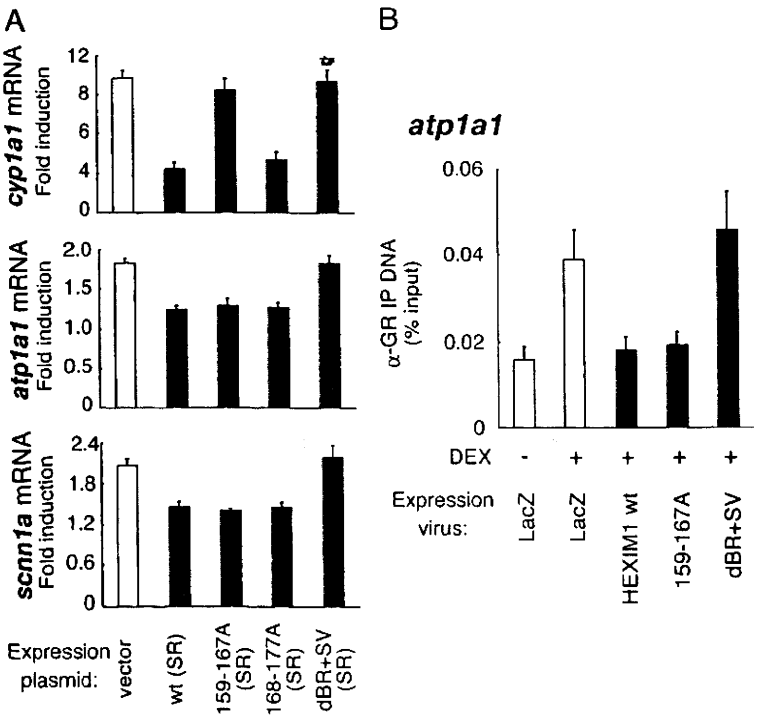

Fig. 8. P-TEFb Is Not Involved in HEXIM1-Mediated Suppression of Glucocorticoid Responsiveness

A, HeLa cells were transfected with 3 μg of empty vector or expression plasmids for the indicated HA-tagged mutant HEXIM1. Cells were infected 4 h later with AdsiHEXIM1 in phenol red-free Opti-MEM I at MOI of 100 for 36 h, and treated with vehicle or 10 nM 3MC (*top panel*), or 100 nM DEX (*middle* and *bottom panels*) for 6 h. Endogenous mRNA levels for *cyp1a1*, *atp1a1*, *scnn1a*, and *gapdh* were measured with qRT-PCR. Samples were normalized to *gapdh* mRNA, and mRNA induction levels by cognate ligands are shown in fold induction. *Error bars* represent SD values of at least three independent experiments. B, HepG2 cells were infected with FLAG-GR-expressing adenovirus (MOI of 50) along with LacZ- or mutant HEXIM1-expressing adenoviruses (MOI of 40) in phenol red-free Opti-MEM I for 24 h, and the cells were treated with 1 μM DEX for 20 min. ChIP assays were performed with polyclonal anti-GR antibodies as described in *Materials and Methods*. *Error bars* represent SD values of at least three independent experiments. IP, Immunoprecipitation.

(Figs. 6B and 7B), suppressed AhR-mediated transcription as well as wild type (SR) (Fig. 8A). These effects of wild-type (SR) and mutant HEXIM1 were also observed in the other P-TEFb-regulated genes depicted in Fig. 5A (data not shown). With respect to GR target genes, mRNA expression of *atp1a1* and *scnn1a* was stimulated by 1.8-fold and 2.1-fold, respectively, in response to 6 h treatment with 100 nM DEX in HEXIM1 knocked-down cells (Fig. 8A). Complementation of wild-type (SR) HEXIM1 or 168–177A (SR) significantly repressed induction of mRNA expression of *atp1a1* and *scnn1a* (Fig. 8A). In contrast to *cyp1a1*, 159–167A (SR) suppressed glucocorticoid-induced enhancement of mRNA expression of *atp1a1* and *scnn1a* comparable to that of wild type (SR) (Fig. 8A), indicating that P-TEFb-binding activity of HEXIM1 is dispensable but GR-binding activity is important for the suppression. Consistently, dBR+SV (SR), which does not bind GR, did not affect mRNA induction of *atp1a1* and *scnn1a* (Fig. 8A). The importance of GR binding of HEXIM1 was also confirmed in ChIP assay. Recombinant adenovirus-mediated expression of 159–167A in HepG2 cells suppressed DEX-dependent recruitment of FLAG-GR onto *atp1a1* promoter, whereas dBR+SV did not (Fig. 8B). Using *scnn1a*, we obtained identical results (data not shown). Taken together, we may conclude that direct interaction between GR and HEXIM1 is critical for HEXIM1-mediated glucocorticoid resistance of *atp1a1* and *scnn1a* in HepG2 cells.

DISCUSSION

As described in the introductory section, HEXIM1 is currently considered to be a multifunctional protein, acting at a specific stage of gene expression. In the present study, we intended to characterize endogenous HEXIM1 function for modulation of GR-mediated transcriptional regulation. For that purpose, we focused on *atp1a1* and *scnn1a*, because expression of these genes is resistant in HEXIM1-rich HepG2 cells to treatment with DEX (Fig. 1A). Treatment with histone deacetylase inhibitor did not result in liberation of these genes in HepG2 cells (Fig. 1A), suggesting that the observed DEX resistance is not due to irreversible alteration in higher order chromatin structure or histone acetylation-related chromatin packaging. In support of this, these genes retain responsiveness to other extracellular stimuli in liver and HepG2 cells (Refs. 10 and 11 and data not shown). We showed that, in HepG2 cells, knockdown of HEXIM1 by siRNA not only canceled the DEX resistance but also rather enhanced DEX-responsive mRNA expression of these genes (Fig. 3C). Moreover, our ChIP assay clearly demonstrated that siRNA-mediated knockdown of HEXIM1 restored hormone-dependent GR recruitment onto the promoters of those genes in parallel with corresponding increase in RNAPII binding (Fig. 3D). Such effect of reduction in endogenous HEXIM1 level was mimicked by exogenous overexpression of GR (Fig. 4, B and C), indicating that GR-HEXIM1 ratio could be a determinant of glucocorticoid resistance/sensitivity of those genes. As anticipated, overexpression of HEXIM1 turned those promoters more or less resistant to DEX (Fig. 4B).

Endogenous HEXIM1 seems to negatively modulate all GR target genes but not completely diminish DEX responsiveness of all of them in HepG2 cells (Fig. 4), indicating that efficiency of the suppression by HEXIM1 is dependent on gene context. Indeed, our previous DNA microarray analyses showed that the extent of reducing DEX responsiveness by overexpressed HEXIM1 was variable among different genes in HepG2 cells (30). It is also reported that GRE occupancy with GR in alveolar epithelial A549 cells is generally restricted to such genes that are actually regulated by glucocorticoids in those cells (21). This observation strongly supports the idea that gene-specific determination of GR recruitment to GRE is important in tissue-specific regulation of glucocorticoid-responsive gene expression at the level preceding transcription initiation. We recently demonstrated that HEXIM1 directly binds GR and that GR or other oxosteroid receptors are preferential partners of HEXIM1 (48). In this line, we might speculate that HEXIM1 squelches GR in the nucleus and inhibits its access to target gene promoter, and such negative effect of HEXIM1 is, more or less, shared by many genes. Some GR-target genes, including *atp1a1* and *scnn1a*, therefore, might be particularly susceptible to HEXIM1 and resistant to glucocorticoids in HEXIM1-rich cells, *i.e.* HepG2 cells. Certain promoters, *e.g. sgk1* promoter, allow hormone-dependent GR binding in HepG2 cells, strongly supporting the notion that promoter recruitment of GR is determined in a gene context-dependent manner as previously predicted in other GR-regulated genes (56).

We also revealed that P-TEFb-suppressing activity of HEXIM1 is not prerequisite for glucocorticoid resistance of these genes (Fig. 8A). Furthermore, the fact that 159–167A binds GR and suppresses GR recruitment to the target gene (Figs. 6C and 8B) again emphasizes the importance of the suppression of GR recruitment through direct GR-HEXIM1 interaction in the mechanisms of glucocorticoid resistance by HEXIM1. These results highlighted the role of HEXIM1 in P-TEFb-independent and gene-selective suppression of mRNA expression. The bimodal roles of HEXIM1 may differentially contribute to suppressing mRNA expression in a gene context-dependent manner. In this line, it should be noted that other transcription factors, such as estrogen receptor (45) and CCAAT/enhancer binding protein α (44), which were shown to directly interact with HEXIM1, may also be controlled by HEXIM1 through a P-TEFb-independent mechanism. Moreover, the interaction of HEXIM1 with these transcription factors has been shown to be a molecular basis for various physiological or pathological actions of HEXIM1 (44, 45).

Our previous observation revealed that HEXIM1/GR complexes reside in a distinct subnuclear area (30). Given this, HEXIM1 might prevent intranuclear GR from accessing to the promoter and decrease the amount of available GR for transcription. Since we revealed that the central and C-terminal regions of HEXIM1 are indispensable for its proper nuclear localization and GR repression (Refs. 41 and 48, and data not shown), HEXIM1 might anchor at as yet unknown but saturable subnuclear structure via these regions. Increasing evidence indicates that the C-terminal region of HEXIM1 possesses various functions, *e.g.* P-TEFb-binding (57, 58), self-oligomerization (59–61), and interaction with transcription factors (44, 51). Recently, nucleophosmin was shown to bind HEXIM1 via BR and promote its degradation (62). Taken together, it may be indicated that subnuclear localization and function of HEXIM1 might be tightly controlled via multimodal interactions among distinct HEXIM1 domains and various nuclear machineries to elicit fine tuning of transcriptional control of gene expression. In any case, an important question to be solved is how multiple functions of HEXIM1 are rationally regulated in a gene- or tissue-dependent manner.

Expression levels of HEXIM1 vary in different tissues and are modulated during differentiation and development as well as in response to extracellular stimuli (see "Introduction"). Disturbances of tissue-specific glucocorticoid responses have been implicated in pathophysiology of rheumatoid arthritis, osteoarthritis, Crohn's disease, ulcerative colitis, asthma, AIDS, osteoporosis, and metabolic syndromes (63). Numbers of proteins have been shown to affect GR activity at different steps of GR signaling pathway and indicated to be potentially involved in the pathogenesis of such diseases that have relations to disturbed glucocorticoid responses in particular tissues (63). HEXIM1-mediated repression of GR might be one of such mechanisms and play pathological roles in certain diseases. On the other hand, glucocorticoids are still indispensable in treatment for a numerous diseases (4, 64). However, the desired therapeutic effects are often accompanied by severe side effects. Pharmacological alteration of the expression levels of HEXIM1, if possible, might indirectly modulate glucocorticoid effects in a tissue-specific manner and enable selective expression of pharmacological actions of glucocorticoids in given tissues. Along with development of selective GR modulators (26), HEXIM1 might also be considered as a drug target for tissue-specific modulation of GR actions.

MATERIALS AND METHODS

Reagents and Antibodies

DEX, troglitazone (TGZ), 3MC, and TSA were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IL-6 was from Peprotech (London, UK). Other reagents were from Nacalai Tesque (Kyoto, Japan) unless otherwise specified. Polyclonal antibodies against CDK9, STAT3, HSF1, CycT1, PPARγ, GR, and HA-peptide were from Santa Cruz Biotechnology, Inc. (sc-484, sc-7179, sc-9144, sc-8127, sc-7196, sc-8992, and sc-805, respectively; Santa Cruz, CA). Polyclonal anti-AhR antibodies were from Biomol (SA-210; Plymouth Meeting, PA). Polyclonal anti-FLAG-peptide antibodies and monoclonal anti-α-actinin antibody were from Sigma-Aldrich (F7425 and A5044, respectively). Monoclonal anti-GR antibody was from BD Biosciences (San Jose, CA). Monoclonal anti-RNAPII antibody was from Covance Laboratories, Inc. (MMS-126R; Princeton, NJ). Rabbit antihuman HEXIM1 antiserum and rabbit antimouse HEXIM1 antiserum were generated against a peptide corresponding from 39–53 amino acids of human HEXIM1 (RVPEEDSRWQSRAFP) and 55–69 amino acids of mouse HEXIM1 (SGSRPGQEGEGGLKH), respectively. Polyclonal anti-HEXIM1 affinity-purified antibodies were obtained from antihuman HEXIM1 antiserum with immunogen-immobilized affinity matrix (Kitayama Labes, Ina, Japan).

Cell Culture and Transfection

HepG2, 293, COS7, and HeLa cells were from RIKEN cell bank (Tsukuba, Japan) and maintained in DMEM supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and antibiotics in a humidified atmosphere at 37 C with 5% CO_2. Before transfection, cells were washed twice with PBS, and media were replaced with phenol red-free Opti-MEM I (Invitrogen). Transient transfection was performed using TransIt-LT1 transfection reagent (Panvera, Madison, WI) as described previously (65). Total amounts of plasmids to transfect were kept constant by adding empty vector.

Western Blot Analysis

Whole-cell extracts or nuclear extracts were prepared as described previously (30), resolved in sodium dodecyl sulfate (SDS)-polyacrylamide gels, and blotted to polyvinylidene fluoride membranes. The membranes were incubated with Blocking One (Nacalai Tesque) at room temperature for 1 h, incubated with specific antibodies diluted in Blocking One (1:500 dilution for HA-peptide or 1:2000 for the others) at 4 C for 18 h, and then, washed three times with TBS-T (25 mM Tris-HCl, pH 8.0; 125 mM NaCl; 0.1% Tween 20), incubated with secondary antibodies conjugated to horseradish peroxidase (GE Healthcare, Buckinghamshire, UK) at room temperature for 30 min, washed three times with TBS-T, and detected with Chemi-Lumi One L (Nacalai Tesque) according to manufacturer's instruction.

Recombinant DNA and Adenoviruses

Expression plasmids for FLAG-tagged HEXIM1 (wild-type and dBR+SV) were described previously (30). pFLAG-CMV2-derived mammalian expression plasmids for mutant FLAG-HEXIM1 (159–167A and 168–177A) were generous gifts from Dr. Q. Zhou (University of California, Berkeley, CA). cDNA fragments for wild-type (SR), 159–167A (SR), 168–177A (SR), and dBR+SV (SR) HEXIM1 were generated by a standard PCR protocol using custom-designed primers and subcloned into pCMV-HA (TaKaRa, Otsu, Japan) or pFLAG-CMV2 (Sigma-Aldrich) expression plasmid using blunt-ended *Eco*RI and *Xho*I sites. The expression plasmid for human PPARγ, pCMX-6His-PPARγ, was generated by cloning appropriate PCR fragments into pCMX-6His vector (65). The PPAR response element (PPARRE)-driven reporter plasmid p3xPPARRE-LUC was a kind gift from Dr. E. A. Jansson (Karolinska Institutet, Stockholm, Sweden). All plasmids constructed above were verified by DNA sequencing. Recombinant adenoviruses encoding double-stranded hairpin RNAs for siRNA

against HEXIM1, AdsiHEXIM1, or control siRNA, Adsictrl, were constructed by subcloning expression cassettes from pSilencer3.1-H1 neo-derived expression plasmids (30) into adenoviral genome using Adenovirus Expression Vector Kit (TaKaRa) according to the manufacturer's instruction. Recombinant adenoviruses prepared from 293 cells were purified with Virakit AdenoMini-24 (Virapur, San Diego, CA) and titrated using Adeno-X Rapid Titer Kit (TaKaRa).

qRT-PCR

Total RNA was prepared with Sepasol-RNA I super (Nacalai Tesque), reverse-transcribed with oligo-dT primer using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). qRT-PCR was performed with the LightCycler TaqMan Master, Universal ProbeLibrary Set, Human, and LightCycler ST300 systems (Roche, Indianapolis, IN) according to manufacturer's instructions. Expression levels of mRNA were calculated on the basis of standard curves generated for each gene. mRNA for *gapdh* was used as an internal control. Sequences of primers used in this study are shown below:

atp1a1: 5′-ccctggctgctttccttt-3′ and 5′-ggcacagaaccacc-aggta-3′

scnn1a: 5′-aaccaggtctcctgcaacc-3′ and 5′-gaaagtatag-cagtttccatacatcg-3′

sgk1: 5′-cctgagcttatgaatgccaac-3′ and 5′-gccaaggttg-atttgctgag-3′

adh1a: 5′-aaggcccatgaagttcgtatt-3′ and 5′-ccacgtggt-catctgtgc-3′

cyp1a1: 5′-cccagctcagctcagtacct-3′ and 5′-ggagattggg-aaaagcatga-3′

junb: 5′-atacacagctacgggatacgg-3′ and 5′-gctcggtttca-ggagtttgt-3′

hspa1a: 5′-ggagtcctacgccttcaaca-3′ and 5′-ccagcacctt-cttcttgtcg-3′

fos: 5′-ctaccactcacccgcagact-3′ and 5′-aggtccgtgcaga-agtcct-3′

gapdh: 5′-agccacatcgctcagaca-3′ and 5′-gcccaatacgacc-aaatcc-3′

ChIP

ChIP assay was performed with ChIP Assay Kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer's instructions with minor modification. First, HepG2 cells were cultured in phenol red-free Opti-MEM I for 24 h for hormone depletion. Then, the cells were treated with 1 μM DEX or 0.1% ethanol (vehicle) for the indicated time periods. After treatment, the cells were cross-linked in 1% formaldehyde for 10 min at 37 C. Cross-linking was stopped with addition of glycine to medium to a final 125 mM for 5 min at 37 C, after which the cells were rinsed with ice-cold PBS twice and harvested. Cell pellets were collected and resuspended in SDS-lysis buffer (50 mM Tris, pH 8.0; 1% SDS; 10 mM EDTA; 1 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; 800 nM aprotinin; 15 μM E-64; 20 μM leupeptin-hemisulfate; 50 μM bestatin; and 10 μM pepstatin A) for 10 min at 4 C. Chromatin was sheared to an average size of 500 bp by sonication of the lysate using a Bioruptor Ultrasonicator (Cosmo-Bio, Tokyo, Japan). Lysates corresponding to 2×10^6 cells were diluted 10-fold in ChIP dilution buffer (0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris, pH 8.1; and 167 mM NaCl) and precleared with Salmon Sperm DNA/Protein A Agarose beads (Upstate Biotechnology) at 4 C for 30 min. Supernatants were then collected and incubated with 5 μg of anti-GR polyclonal antibodies or anti-RNAPII monoclonal antibody at 4 C overnight. To collect immune complex, Salmon Sperm DNA/Protein A Agarose beads were added and further incubated at 4 C for 1 h. The beads were then washed twice each with Low-Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris, pH 8.1; and 150 mM NaCl), High-Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris, pH 8.1; and 500 mM NaCl), LiCl Immune Complex Wash Buffer (0.25 M LiCl; 1% Nonidet P-40; 1% deoxycholate; 1 mM EDTA; and 10 mM Tris, pH 8.1), and Tris-EDTA buffer. Protein-chromatin complex was eluted with elution buffer (10 mM dithiothreitol, 1% SDS, and 0.1 M $NaHCO_3$), and reversal of cross-link of eluates was performed in 200 mM NaCl at 65 C for 6 h, after which proteins were digested with proteinase K at 45 C for 1 h. Precipitated DNA fragments were recovered by QIAquick DNA purification kit (QIAGEN, Chatsworth, CA) and quantified with qRT-PCR using appropriate primer sets. Sequences of primers used in this study are shown below:

sgk1 −1238: 5′-acctcctcacgtgttcttgg-3′ and *sgk1* −982: 5′-caagcaaggctgaaaaatcc-3′ for GR

sgk1 −173: 5′-cctctcaatggggacagaac-3′ and *sgk1* +85: 5′-ccttagcagcctcagttttca-3′ for RNAPII

atp1a1 −732: 5′-cgcccttcagattctcattt-3′ and *atp1a1* −447: 5′-ggactcagggatgctgga-3′ for GR

atp1a1+156: 5′-ccctagctccctccacttg-3′ and *atp1a1* +239: 5′-tcgctggagaatcagagagaa-3′ for RNAPII

FLAG-Affinity Purification

HeLa cells (2.5×10^6) were transfected with 4 μg of pFLAG-CMV2-derived expression plasmids. After 4 h, media were replaced with DMEM supplemented with 10% fetal calf serum. After 32 h, cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (wt/vol) Nonidet P-40, 1 μM dithiothreitol, 0.5 μM phenylmethylsulfonyl fluoride], centrifuged at 20,000 × *g* for 20 min. Supernatant was diluted in FAR buffer [16.7 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.33% (wt/vol) Nonidet P-40, 0.33 μM dithiothreitol, 0.17 μM phenylmethylsulfonyl fluoride], applied to anti-FLAG M2-agarose beads (Sigma-Aldrich), incubated for 2 h at room temperature. The beads were washed three times with FAR buffer. Bound proteins were eluted with SDS-sample loading buffer and subjected to Western blot analysis using anti-FLAG peptide, anti-CycT1, and anti-CDK9 antibodies.

Luciferase Assay

COS7 cells (1×10^6) were transfected with 2 μg of reporter plasmids (p2xGRE-LUC or p3xPPARRE-LUC), 2.5 ng of expression plasmids for the receptors (pCMX-6His-GR or pCMX-6His-PPARγ), and pCMV-HA-derived HEXIM1 expression plasmids. After 4 h, media were replaced with fresh phenol red-free Opti-MEM I, and infected with recombinant adenoviruses at multiplicity of infection (MOI) of 100. After 20 h, cells were treated with 100 nM DEX, 100 nM TGZ or vehicle (0.1% ethanol), and further cultured for 18 h. Cells were lysed in Cell Culture Lysis Reagent (Promega Corp., Madison, WI), and cellular luciferase activity was measured by using Luciferase Assay System (Promega). Relative light units were normalized to the protein amounts determined with BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL).

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REFERENCES

1. Duma D, Jewell CM, Cidlowski JA 2006 Multiple glucocorticoid receptor isoforms and mechanisms of post-translational modification. J Steroid Biochem Mol Biol 102:11–21
2. Beato M 1989 Gene regulation by steroid hormones. Cell 56:335–344
3. Sapolsky RM, Romero LM, Munck AU 2000 How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr Rev 21:55–89
4. Rhen T, Cidlowski JA 2005 Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. N Engl J Med 353:1711–1723
5. Lu NZ, Cidlowski JA 2004 The origin and functions of multiple human glucocorticoid receptor isoforms. Ann NY Acad Sci 1024:102–123
6. Lingrel JB, Orlowski J, Shull MM, Price EM 1990 Molecular genetics of Na,K-ATPase. Prog Nucleic Acid Res Mol Biol 38:37–89
7. Bens M, Chassin C, Vandewalle A 2006 Regulation of NaCl transport in the renal collecting duct: lessons from cultured cells. Pflugers Arch 453:133–146
8. Garty H, Palmer LG 1997 Epithelial sodium channels: function, structure, and regulation. Physiol Rev 77: 359–396
9. Bonny O, Hummler E 2000 Dysfunction of epithelial sodium transport: from human to mouse. Kidney Int 57: 1313–1318
10. Gick GG, Ismail-Beigi F, Edelman IS 1988 Thyroidal regulation of rat renal and hepatic Na,K-ATPase gene expression. J Biol Chem 263:16610–16618
11. Ismail-Beigi F, Pressley TA, Haber RS, Gick GG, Loeb JN, Edelman IS 1988 Kinetic analysis of Na,K-activated adenosine triphosphatase induced by low external K+ in a rat liver cell line. J Biol Chem 263:8162–8167
12. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM 1995 The nuclear receptor superfamily: the second decade. Cell 83:835–839
13. Beato M, Herrlich P, Schutz G 1995 Steroid hormone receptors: many actors in search of a plot. Cell 83: 851–857
14. Belandia B, Parker MG 2003 Nuclear receptors: a rendezvous for chromatin remodeling factors. Cell 114: 277–280
15. Lonard DM, O'Malley B W 2007 Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. Mol Cell 27:691–700
16. Kumar R, Thompson EB 2005 Gene regulation by the glucocorticoid receptor: structure:function relationship. J Steroid Biochem Mol Biol 94:383–394
17. Lu NZ, Wardell SE, Burnstein KL, Defranco D, Fuller PJ, Giguere V, Hochberg RB, McKay L, Renoir JM, Weigel NL, Wilson EM, McDonnell DP, Cidlowski JA 2006 International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Rev 58:782–797
18. Woychik NA, Hampsey M 2002 The RNA polymerase II machinery: structure illuminates function. Cell 108: 453–463
19. Kadonaga JT 2004 Regulation of RNA polymerase II transcription by sequence-specific DNA binding factors. Cell 116:247–257
20. Phuc Le P, Friedman JR, Schug J, Brestelli JE, Parker JB, Bochkis IM, Kaestner KH 2005 Glucocorticoid receptor-dependent gene regulatory networks. PLoS Genet 1:e16
21. So AY, Chaivorapol C, Bolton EC, Li H, Yamamoto KR 2007 Determinants of cell- and gene-specific transcriptional regulation by the glucocorticoid receptor. PLoS Genet 3:e94
22. Wu W, Chaudhuri S, Brickley DR, Pang D, Karrison T, Conzen SD 2004 Microarray analysis reveals glucocorticoid-regulated survival genes that are associated with inhibition of apoptosis in breast epithelial cells. Cancer Res 64:1757–1764
23. Rogatsky I, Wang JC, Derynck MK, Nonaka DF, Khodabakhsh DB, Ha CM, Darimont BD, Garabedian MJ, Yamamoto KR 2003 Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. Proc Natl Acad Sci USA 100:13845–13850
24. Tomlinson JW, Walker EA, Bujalska IJ, Draper N, Lavery GG, Cooper MS, Hewison M, Stewart PM 2004 11β-Hydroxysteroid dehydrogenase type 1: a tissue-specific regulator of glucocorticoid response. Endocr Rev 25: 831–866
25. Lonard DM, O'Malley BW 2005 Expanding functional diversity of the coactivators. Trends Biochem Sci 30: 126–132
26. Lu NZ, Cidlowski JA 2006 Glucocorticoid receptor isoforms generate transcription specificity. Trends Cell Biol 16:301–307
27. Kusuhara M, Nagasaki K, Kimura K, Maass N, Manabe T, Ishikawa S, Aikawa M, Miyazaki K, Yamaguchi K 1999 Cloning of hexamethylene-bis-acetamide-inducible transcript, HEXIM1, in human vascular smooth muscle cells. Biomed Res 20:273–279
28. Michels AA, Nguyen VT, Fraldi A, Labas V, Edwards M, Bonnet F, Lania L, Bensaude O 2003 MAQ1 and 7SK RNA interact with CDK9/cyclin T complexes in a transcription-dependent manner. Mol Cell Biol 23: 4859–4869
29. Yik JH, Chen R, Nishimura R, Jennings JL, Link AJ, Zhou Q 2003 Inhibition of P-TEFb (CDK9/Cyclin T) kinase and RNA polymerase II transcription by the coordinated actions of HEXIM1 and 7SK snRNA. Mol Cell 12:971–982
30. Shimizu N, Ouchida R, Yoshikawa N, Hisada T, Watanabe H, Okamoto K, Kusuhara M, Handa H, Morimoto C, Tanaka H 2005 HEXIM1 forms a transcriptionally abortive complex with glucocorticoid receptor without involving 7SK RNA and positive transcription elongation factor b. Proc Natl Acad Sci USA 102:8555–8560
31. Sims RJ, 3rd, Belotserkovskaya R, Reinberg D 2004 Elongation by RNA polymerase II: the short and long of it. Genes Dev 18:2437–2468
32. Malik S, Roeder RG 2005 Dynamic regulation of pol II transcription by the mammalian Mediator complex. Trends Biochem Sci 30:256–263
33. Peterlin BM, Price DH 2006 Controlling the elongation phase of transcription with P-TEFb. Mol Cell 23:297–305
34. Karn J 1999 Tackling Tat. J Mol Biol 293:235–254
35. Barboric M, Nissen RM, Kanazawa S, Jabrane-Ferrat N, Peterlin BM 2001 NF-κB binds P-TEFb to stimulate transcriptional elongation by RNA polymerase II. Mol Cell 8:327–337
36. Giraud S, Hurlstone A, Avril S, Coqueret O 2004 Implication of BRG1 and cdk9 in the STAT3-mediated activation of the p21waf1 gene. Oncogene 23:7391–7398

37. Boehm AK, Saunders A, Werner J, Lis JT 2003 Transcription factor and polymerase recruitment, modification, and movement on dhsp70 in vivo in the minutes following heat shock. Mol Cell Biol 23:7628–7637
38. Tian Y, Ke S, Chen M, Sheng T 2003 Interactions between the aryl hydrocarbon receptor and P-TEFb. Sequential recruitment of transcription factors and differential phosphorylation of C-terminal domain of RNA polymerase II at cyp1a1 promoter. J Biol Chem 278: 44041–44048
39. Zhou Q, Yik JH 2006 The Yin and Yang of P-TEFb regulation: implications for human immunodeficiency virus gene expression and global control of cell growth and differentiation. Microbiol Mol Biol Rev 70:646–659
40. Dey A, Chao SH, Lane DP 2007 HEXIM1 and the control of transcription elongation: from cancer and inflammation to AIDS and cardiac hypertrophy. Cell Cycle 6:1856–1863
41. Ouchida R, Kusuhara M, Shimizu N, Hisada T, Makino Y, Morimoto C, Handa H, Ohsuzu F, Tanaka H 2003 Suppression of NF-κB-dependent gene expression by a hexamethylene bisacetamide-inducible protein HEXIM1 in human vascular smooth muscle cells. Genes Cells 8:95–107
42. Wittmann BM, Wang N, Montano MM 2003 Identification of a novel inhibitor of breast cell growth that is down-regulated by estrogens and decreased in breast tumors. Cancer Res 63:5151–5158
43. Haaland RE, Herrmann CH, Rice AP 2005 siRNA depletion of 7SK snRNA induces apoptosis but does not affect expression of the HIV-1 LTR or P-TEFb-dependent cellular genes. J Cell Physiol 205:463–470
44. Montano MM, Doughman YQ, Deng H, Chaplin L, Yang J, Wang N, Zhou Q, Ward NL, Watanabe M 2008 Mutation of the HEXIM1 gene results in defects during heart and vascular development partly through downregulation of vascular endothelial growth factor. Circ Res 102: 415–422
45. Wittmann BM, Fujinaga K, Deng H, Ogba N, Montano MM 2005 The breast cell growth inhibitor, estrogen down regulated gene 1, modulates a novel functional interaction between estrogen receptor alpha and transcriptional elongation factor cyclin T1. Oncogene 24:5576–5588
46. Li Q, Cooper JJ, Altwerger GH, Feldkamp MD, Shea MA, Price DH 2007 HEXIM1 is a promiscuous double-stranded RNA-binding protein and interacts with RNAs in addition to 7SK in cultured cells. Nucleic Acids Res 35: 2503–2512
47. Huang F, Wagner M, Siddiqui MA 2004 Ablation of the CLP-1 gene leads to down-regulation of the HAND1 gene and abnormality of the left ventricle of the heart and fetal death. Mech Dev 121:559–572
48. Yoshikawa N, Shimizu N, Sano M, Ohnuma K, Iwata S, Hosono O, Fukuda K, Morimoto C, Tanaka H 2008 Role of the hinge region of glucocorticoid receptor for HEXIM1-mediated transcriptional repression. Biochem Biophys Res Commun 371:44–49
49. Shull MM, Pugh DG, Lingrel JB 1990 The human Na, K-ATPase α 1 gene: characterization of the 5′-flanking region and identification of a restriction fragment length polymorphism. Genomics 6:451–460
50. Itani OA, Liu KZ, Cornish KL, Campbell JR, Thomas CP 2002 Glucocorticoids stimulate human sgk1 gene expression by activation of a GRE in its 5′-flanking region. Am J Physiol Endocrinol Metab 283:E971–E979
51. Kohoutek J, Blazek D, Peterlin BM 2006 Hexim1 sequesters positive transcription elongation factor b from the class II transactivator on MHC class II promoters. Proc Natl Acad Sci USA 103:17349–17354
52. Aida M, Chen Y, Nakajima K, Yamaguchi Y, Wada T, Handa H 2006 Transcriptional pausing caused by NELF plays a dual role in regulating immediate-early expression of the junB gene. Mol Cell Biol 26:6094–6104
53. Ishikawa T, Igarashi T, Hata K, Fujita T 1999 c-fos induction by heat, arsenite, and cadmium is mediated by a heat shock element in its promoter. Biochem Biophys Res Commun 254:566–571
54. Ni Z, Schwartz BE, Werner J, Suarez JR, Lis JT 2004 Coordination of transcription, RNA processing, and surveillance by P-TEFb kinase on heat shock genes. Mol Cell 13:55–65
55. Yik JH, Chen R, Pezda AC, Samford CS, Zhou Q 2004 A human immunodeficiency virus type 1 Tat-like arginine-rich RNA-binding domain is essential for HEXIM1 to inhibit RNA polymerase II transcription through 7SK snRNA-mediated inactivation of P-TEFb. Mol Cell Biol 24:5094–5105
56. Starr DB, Matsui W, Thomas JR, Yamamoto KR 1996 Intracellular receptors use a common mechanism to interpret signaling information at response elements. Genes Dev 10:1271–1283
57. Schulte A, Czudnochowski N, Barboric M, Schonichen A, Blazek D, Peterlin BM, Geyer M 2005 Identification of a cyclin T-binding domain in Hexim1 and biochemical analysis of its binding competition with HIV-1 Tat. J Biol Chem 280:24968–24977
58. Dames SA, Schonichen A, Schulte A, Barboric M, Peterlin BM, Grzesiek S, Geyer M 2007 Structure of the Cyclin T binding domain of Hexim1 and molecular basis for its recognition of P-TEFb. Proc Natl Acad Sci USA 104: 14312–14317
59. Dulac C, Michels AA, Fraldi A, Bonnet F, Nguyen VT, Napolitano G, Lania L, Bensaude O 2005 Transcription-dependent association of multiple positive transcription elongation factor units to a HEXIM multimer. J Biol Chem 280:30619–30629
60. Barboric M, Kohoutek J, Price JP, Blazek D, Price DH, Peterlin BM 2005 Interplay between 7SK snRNA and oppositely charged regions in HEXIM1 direct the inhibition of P-TEFb. EMBO J 24:4291–4303
61. Blazek D, Barboric M, Kohoutek J, Oven I, Peterlin BM 2005 Oligomerization of HEXIM1 via 7SK snRNA and coiled-coil region directs the inhibition of P-TEFb. Nucleic Acids Res 33:7000–7010
62. Gurumurthy M, Tan CH, Ng R, Zeiger L, Lau J, Lee J, Dey A, Philp R, Li Q, Lim TM, Price DH, Lane DP, Chao SH 2008 Nucleophosmin interacts with HEXIM1 and regulates RNA polymerase II transcription. J Mol Biol 378: 302–317
63. Kino T, De Martino MU, Charmandari E, Mirani M, Chrousos GP 2003 Tissue glucocorticoid resistance/hypersensitivity syndromes. J Steroid Biochem Mol Biol 85: 457–467
64. Tanaka H, Yoshikawa N, Shimizu N, Morimoto C 2004 Selective modulation of glucocorticoid receptor function toward development of novel anti-inflammation: lessons from a phenyl-pyrazolo-steroid cortivazol. Mod Rheumatol 14:347–355
65. Yoshikawa N, Yamamoto K, Shimizu N, Yamada S, Morimoto C, Tanaka H 2005 The distinct agonistic properties of the phenylpyrazolosteroid cortivazol reveal inter-domain communication within the glucocorticoid receptor. Mol Endocrinol 19:1110–1124

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Activation of Mitochondrial Biogenesis by Hormesis

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Mitochondria play a major role in oxidative energy production, reduction–oxidation reaction (redox) control and calcium homeostasis. Although mitochondria contain DNA with mitochondrial-specific genes, most mitochondrial proteins are encoded by the nDNA, synthesized in the cytosol, and imported into mitochondria. The expression of nuclear genes that encode mitochondrial proteins that function in metabolic pathways such as the trichloroacetic acid cycle (TCA), oxidative phosphorylation, heme synthesis, and in mitochondrial DNA replication and transcription (eg, mitochondrial transcription factor A [Tfam]), is coordinately regulated by the transcriptional coactivators PPARγ coactivator (PGC)-1α and PGC-1β through activation of nuclear respiratory factor (NRF)-1 and NRF-2.[1]

In their recent publications, Piantadosi et al provided insight into the mechanisms underlying the interaction between mitochondria-derived reactive oxygen species (ROS) signaling and mitochondrial biogenesis. First, lipid hydroperoxide regulates Tfam expression through phosphorylation of NRF-1 via Akt activation, which promotes nuclear translocation of NRF-1 and binding to the Tfam promoter.[2] Second, carbon monoxide (CO) induced mitochondrial biogenesis via activation of Akt1/PKB and guanylate cyclase, which augmented gene and protein expression of NRF-1 and NRF-2, PGC-1α, and TFAM.[3] CO-induced mitochondrial ROS result in the activation of AKT. Third, the anthracycline anticancer agent doxorubicin suppresses the nuclear program for mitochondrial biogenesis, and its associated intrinsic antiapoptosis proteins, leading to severe mitochondrial DNA (mtDNA) depletion and apoptosis. CO inhalation or heme oxygenase (Hmo)1 overexpression prevented doxorubicin-induced mtDNA depletion and apoptosis via activation of AKT and guanylate cyclase.[4] Lastly, new work in this issue of *Circulation Research*[5] sheds light on the role of NF-E2–related factor (Nrf)2 as a key transcriptional regulator in mitochondrial ROS-dependent induction of NRF-1 mRNA.

There is increasing evidence to suggest that ROS may be a double-edged sword: although they can be toxic to cells, they may also play an important role in cell signaling involved in the antioxidant defense network. ROS are generated from many sources including the Nox family of NADPH oxidases, xanthine oxidase, and mitochondria, where ROS are produced as a byproduct of oxidative energy production. ROS are very unstable and cannot penetrate lipid membranes; they are therefore retained within the compartment in which they are produced. However, ROS can attack neighboring polyunsaturated fatty acids of the membrane and trigger a chain reaction of lipid peroxidation, resulting in the generation of lipid hydroperoxides and α, β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (4-HNE) (Figure). They are highly electrophilic and react with biomolecules, such as proteins and nucleic acids, generating various adducts. By virtue of their increased chemical stability, these lipid peroxidation products can diffuse greater distances compared with their precursor ROS and can propagate and amplify oxidative injury. Thus, lipid peroxidation products have been implicated in the development and progression of a variety of pathological events such as oxidization of LDL, atherosclerosis, ischemia/reperfusion injury, Alzheimer's disease, cancers, and cell senescence.

However cells are able to sense macromolecular damage and counteract stress-induced damage to reestablish homeostasis. Electrophilic lipid peroxidation products can trigger a cascade of stress resistant pathways in both a tissue- and cell type–specific manner. The induction of stress-protective mechanisms by stress is referred to as "stress-response hormesis."[6] The principle of stress-response hormesis can be seen in many contexts. For example, the ninja, a group of spies and assassins in feudal Japan, were known to regularly take sublethal doses of poison to build their capacity to detoxify xenobiotics and thus protect themselves against assassination with poison. In cell culture, 4-HNE kills cells at a high dose, whereas pretreatment of cells with low-dose 4-HNE upregulates endogenous antioxidant and phase II enzymes, conferring greater tolerance against subsequent oxidative insult.[7] An effect of stress-response hormesis may also be seen in clinical studies that have tested antioxidant supplements for prevention of cardiovascular events[4] and cancers[5] based on the principle that they should prevent oxidative stress-induced macromolecular damage. In both clinical studies, antioxidant supplements may have failed to identify a beneficial effect because this inevitably attenuates the cell-signaling pathways necessary for protection against oxidative stress and reestablishment of redox homeostasis.[6]

Following the induction of oxidative stress, 2 basic leucine zipper transcription factors, Nrf2 and activating transcription factor (ATF)4, are activated at the posttranscriptional level and induce the expression of genes encoding proteins that function as antioxidants and enzymes involved in phase II detoxification and glutathione biosynthesis. Under nonstressed conditions, Nrf2 is tethered in the cytoplasm by Keap1. This complex directs Nrf2 polyubiquitination and degradation. On oxidative stress, Nrf2 is liberated from

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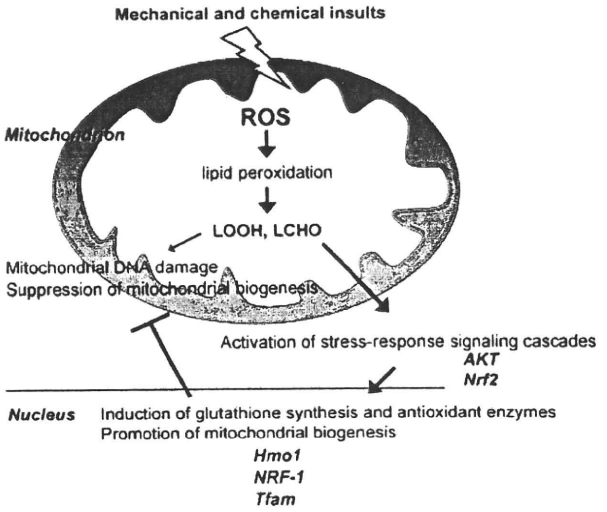

Figure. The generation of ROS and the subsequent oxidative modification of biomolecules are inevitable events in aerobic organisms. The polyunsaturated fatty acids in membrane lipids are particularly vulnerable to ROS attack and they undergo peroxidation. These lipid peroxidation products form protein and DNA adducts and have biphasic properties, in that high doses cause overt toxicity, whereas low doses interact with genetic signaling systems that upregulate gene expression to counteract stressor challenges and to re-establish homeostasis. LOOH indicates lipid hydroperoxides; LCHO, lipid aldehydes.

Keap1 and enters the nucleus, where it can form a heterodimer with the small Maf transcription factor Nrf2 to stimulate the expression of antioxidant response element–containing genes, including NAD(P)H:quinine oxidoreductase, heme-oxygenase 1, γ-glutamylcysteine synthetase, glutathione *S*-transferase, glutathione peroxidase, glutathione reductase, cysteine glutamate transporter, and multidrug resistance–associated protein 1.[11] Oxidative stress leads to the phosphorylation of the α subunit of translation initiation factor 2 (eIF2α). Phosphorylation of eIF2α inhibits general protein synthesis but specifically upregulates translation of ATF4. ATF4 forms homodimers and heterodimers with members of the AP-1 and C/EBP family of proteins to regulate the expression of genes involved in amino acid metabolism which provide the precursor amino acids necessary for glutathione biosynthesis, such as phosphoserine amino transferase, phosphoserine phosphatase, cystathione γ-lysate, and methylenetetrafolate dehydrogenase[12] Thus, Nrf2 and ATF4 coordinately regulate glutathione biosynthesis and the glutathione redox cycle.

Intense muscular contractile activity by exercise results in oxidative stress, as indicated by altered muscle and blood glutathione concentrations and increases in protein, DNA, and lipid peroxidation. Interestingly, it was recently reported that excess vitamin C supplements decrease training efficiency via the reduction of the exercise-induced expression of PGC-1, NRF-1, and Tfam.[13] This observation further suggests that ROS cannot only be considered to be toxic byproducts; they also play an important role in the cell signaling that regulates expression of genes involved in mitochondrial biogenesis. Piantadosi et al first demonstrated a role for Nrf2 in ROS-mediated induction of NRF-1. The NRF-1 promoter contains multiple antioxidant response element motifs and mitochondrial-derived ROS enhance Nrf2 binding to the NRF-1 promoter via AKT-mediated derepression of Nrf2 nuclear translocation. In the heart, however, the role of Nrf2 signaling in the basal expression, as well as the induction of antioxidants in pathological circumstances remains unclear.

Mitochondrial DNA copy number and mitochondrial gene expression are reduced in heart failure. Not surprisingly, concomitant downregulation of PGC-1α, NRF-1, and Tfam in the failing heart is observed.[14] The mechanism by which pathophysiological cues downregulate PGC-1/NRF-1/Tfam expression have only begun to be resolved,[15] but it is tempting to speculate that rescue of PGC-1/NRF-1/Tfam expression may have beneficial effects on cardiac function. Indeed, transgenic overexpression of Tfam in the heart ameliorates the decrease in mitochondrial DNA copy number and mitochondrial complex enzyme activities in the hearts and attenuates left ventricular remodeling and failure after myocardial infarction.[16]

The novel concept that mitochondrial biogenesis seems to be triggered by mitochondrial ROS generation is intriguing. To move present knowledge toward more general applicability, the physiological and pathological relevance of mitochondrial ROS-mediated transcriptional and posttranscriptional activation of NRF-1 via AKT, in the setting of postneonatal normal growth, exercise-challenged, pressure-challenged, ischemic, and failing heart need to be clarified.

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Disclosures

None.

References

1. Kelly DP, Scarpulla RC. Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. *Genes Dev.* 2004;18:357–368.
2. Piantadosi CA, Suliman HB. Mitochondrial transcription factor A induction by redox activation of nuclear respiratory factor 1. *J Biol Chem.* 2006;281:324–333.
3. Suliman HB, Carraway MS, Tatro LG, Piantadosi CA. A new activating role for CO in cardiac mitochondrial biogenesis. *J Cell Sci.* 2007;120:299–308.
4. Suliman HB, Carraway MS, Ali AS, Reynolds CM, Welty-Wolf KE, Piantadosi CA. The CO/HO system reverses inhibition of mitochondrial biogenesis and prevents murine doxorubicin cardiomyopathy. *J Clin Invest.* 2007;117:3730–3741.
5. Piantadosi CA, Carraway MS, Babiker A, Suliman HB. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ Res.* 2008;103:1232–1240.
6. Gems D, Partridge L. Stress-response hormesis and aging: "that which does not kill us makes us stronger." *Cell Metab.* 2008;7:200–203.
7. Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y, Osawa T. Activation of stress signaling pathways by the end product of lipid peroxidation. 4-Hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. *J Biol Chem.* 1999;274:2234–2242.
8. Honarbakhsh S, Schachter M. Vitamins and cardiovascular disease. *Br J Nutr.* 2008:1–19.
9. Bjelakovic G, Nikolova D, Simonetti RG, Gluud C. Antioxidant supplements for prevention of gastrointestinal cancers: a systematic review and meta-analysis. *Lancet.* 2004;364:1219–1228.

10. Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, Endo J, Katayama T, Kawamura A, Kohsaka S, Makino S, Ohta S, Ogawa S, Fukuda K. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. *Biochem Biophys Res Commun*. 2008;373:30–35.
11. Motohashi H, Yamamoto M. Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol Med*. 2004;10:549–557.
12. Harding HP, Zhang Y, Zeng H, Novoa I, Lu PD, Calfon M, Sadri N, Yun C, Popko B, Paules R, Stojdl DF, Bell JC, Hettmann T, Leiden JM, Ron D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell*. 2003;11:619–633.
13. Gomez-Cabrera MC, Domenech E, Romagnoli M, Arduini A, Borras C, Pallardo FV, Sastre J, Vina J. Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. *Am J Clin Nutr*. 2008;87:142–149.
14. Garnier A, Fortin D, Delomenie C, Momken I, Veksler V, Ventura-Clapier R. Depressed mitochondrial transcription factors and oxidative capacity in rat failing cardiac and skeletal muscles. *J Physiol*. 2003;551:491–501.
15. Sano M, Wang SC, Shirai M, Scaglia F, Xie M, Sakai S, Tanaka T, Kulkarni PA, Barger PM, Youker KA, Taffet GE, Hamamori Y, Michael LH, Craigen WJ, Schneider MD. Activation of cardiac Cdk9 represses PGC-1 and confers a predisposition to heart failure. *EMBO J*. 2004;23:3559–3569.
16. Ikeuchi M, Matsusaka H, Kang D, Matsushima S, Ide T, Kubota T, Fujiwara T, Hamasaki N, Takeshita A, Sunagawa K, Tsutsui H. Overexpression of mitochondrial transcription factor a ameliorates mitochondrial deficiencies and cardiac failure after myocardial infarction. *Circulation*. 2005;112:683–690.

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