The principal soy phytoestrogen genistein has an array of biological actions. It binds to estrogen receptor (ER) α and β and has ER-mediated estrogenic effects. In addition, it has antiestrogenic effects as well as non-ER-mediated effects such as inhibition of tyrosine kinase. Because of its complex biological actions, the molecular mechanisms of action of genistein are poorly understood. Here we show that genistein dose-dependently increases estrogenic transcriptional activity in mesenchymal progenitor cells, but its biological effects on osteogenesis and adipogenesis are different. At low concentrations (≤1 μM), genistein acts as estrogen, stimulating osteogenesis and inhibiting adipogenesis. At high concentrations (>1 μM), however, genistein acts as a ligand of PPARγ, leading to up-regulation of adipogenesis and down-regulation of osteogenesis. Transfection experiments show that activation of PPARγ by genistein at the micromolar concentrations down-regulates its estrogenic transcriptional activity, while activation of ERα or ERβ by genistein down-regulates PPARγ transcriptional activity. Genistein concurrently activates two different transcriptional factors, ERs and PPARγ, which have opposite effects on osteogenesis or adipogenesis. As a result, the balance between activated ERs and PPARγ determines the biological effects of genistein on osteogenesis and adipogenesis. Our findings may explain distinct effects of genistein in different tissues.

In recent years, soy phytoestrogens have attracted wide attention due to their potential beneficial effects on some common medical disorders (1–3). Genistein, the principal soy phytoestrogen, has an array of biological actions and is widely available in herbal tablets (3–5). It binds to estrogen receptors (ERs), ERα and ERβ, and has ER-mediated effects (6, 7). In addition, it has antiestrogenic effects, but the underlying mechanism is still unknown (1, 2, 4). Non-ER mediated genistein actions such as an inhibition of protein tyrosine kinase, DNA topoisomerases I and II and ribosomal S6 kinase have also been reported (8–10). These actions are most likely mediated through transcriptional processes rather than via direct effects on enzyme activity (11, 12).

Peroxisome proliferator-activated receptor-γ (PPARγ), one of the subtypes of PPARs, is a ligand-dependent transcription factor of the nuclear hormone receptor superfamily (13). PPARγ is most highly expressed in adipose tissue and is involved in critical physiological functions such as adipogenesis and glucose and cholesterol metabolism (14). It is a target for therapeutic intervention in cardiovascular diseases, various cancers, and diabetes (15).

PPARγ is the essential transcriptional factor for adipogenesis (16–18). Adipocytes and the bone-forming cells, the osteoblasts, arise from the same bone marrow mesenchymal precursor cells (19, 20). The osteoprogenitor KS483 cells, which are cloned from mouse calvaria (21, 22), have been shown to differentiate into both osteoblasts and adipocytes. Using this cell line, we recently showed that 17β-estradiol (E2) stimulates osteogenesis and concurrently inhibits adipogenesis in these precursor cells (23). Whether the phytoestrogen genistein has similar effects is unknown.

In the present study, we examined the effects of genistein on osteogenesis and adipogenesis and explored its molecular mechanisms of action. Our results show that genistein, in addition to its estrogenic activity, activates PPARγ, resulting in a down-regulation of osteogenesis and an up-regulation of adipogenesis. This action is concentration-dependent. Our data show that the balance between activated ERs and PPARγ determines the biological effects of genistein.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Assays**—The methods for cell culture have been described before (23). In brief, KS483 cells and mouse bone marrow cells were cultured in phenol red-free α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (Invitrogen) or 15% fetal bovine serum (for mouse bone marrow), 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 10−8 M dexamethasone (only for mouse bone marrow). Cells were continuously exposed to genistein 1 day after plating until the end of the experiment at day 21. Assays for ALP activity and DNA content, mRNA expression by RT-PCR, and Oil-Red-O staining for adipocytes were performed as described previously (23).

**Membrane-bound PPARγ Binding Assay**—Binding assays, using a human full-length PPARγ construct expressed in bacteria, were performed in 96-well plates (24). Binding buffer consisted of 10 mM Tris/HCl, pH 8.2, containing 50 mM KCl and 1 mM diethiothreitol. Membrane preparations (5 μg/ml) were incubated for 180 min at 4 °C in the presence of 1H-rosiglitazone (BRL49653, Amersham Biosciences) (10 nM) and the tested compounds. Nonspecific binding was defined using an excess of unlabelled rosiglitazone (10 μM). Incubation was terminated by the addition of ice-cold 50 mM Tris/HCl buffer, pH 7.4, followed by rapid filtration under reduced pressure through Whatman GF/C filter plates presoaked with ice-cold buffer, followed by three successive
washes with the same buffer. Radioactivity was measured in a Top-Count apparatus (Packard). The receptor preparation used during these experiments presented a $B_{\text{max}}$ of 49 pmol/mg proteins and a $K_d$ of 5.58 nM for [3H]rosiglitazone. Genistein was solubilized in Me$_2$SO and diluted to the appropriate working concentrations (100 nM–0.1 nM).

**Transient Gene Expression Assays in KS483 Cells**—The estrogen-responsive reporter gene construct (2XERE-TATA-luc), which contains two copies of a consensus estrogen response element, and the empty control TATA-luc plasmids were kindly provided by Dr. E. Kalkhoven and Dr. M. G. Parker. The peroxisome proliferator-responsive element (3XPPRE-tk-luc) containing three copies of a consensus peroxisome proliferator-responsive element and the human PPAR$_\gamma$ constructs were kind gifts from Dr. J. Auwerx. The luciferase reporter construct (5XPPRE-TATA-luc) contained five copies of a consensus PPRE and a TATA box and were provided by Dr. M. Karperien. The pT-109 FARE PPRE construct was a kind gift from Dr. K. van der Lee and Dr. M. van Bilsen. The ACO-luc PPRE construct was kindly supplied by Dr. K. W. Kinzler and Dr. B. Vogelstein. The human ER$_\alpha$ construct was kindly provided by Dr. G. Kuiper. KS483 cells were seeded into 24-well plates. After 24 h, they were transfected using a lipid-based FuGENE 6 transfection reagent according to the manufacturer (Roche Molecular Biochemicals). For each triplicate of sample, 100 ng of luciferase reporter and 500 ng of $\beta$-galactosidase expression vector were applied. The transfection medium was changed after 16 h into the different medium as indicated. After 48 h, cells were washed twice with PBS, lysed in PBS containing 1% Triton X-100 and sonicated. Luciferase activity was measured and expressed as fold induction ± S.E., which was corrected for transfection efficiency using $\beta$-galactosidase activity.

**Statistics**—Data are presented as means ± S.E. Differences between groups were accepted at $p < 0.05$, which were assessed by one-way analysis of variance or related test using software Instat.

**RESULTS**

Osteogenesis—As shown in Fig. 1, genistein added to cultures of KS483 cells had a clear biphasic effect on osteogenesis, similar to that of E$_2$ (23). At concentrations from 0.1 to 10 $\mu$M, genistein stimulated ALP activity, nodule formation, and calcium deposition, with a maximal effect at 1 $\mu$M. In contrast, at concentrations of 25 $\mu$M or higher, genistein inhibited ALP activity, nodule formation and Ca$^{2+}$ deposition. These changes were paralleled by mRNA expression of the osteoblastic markers, Cbfa1, osteocalcin, and PTH/PTHrP-R (Fig. 1). Similar stimulatory and inhibitory effects of genistein on bone formation were also observed in mouse bone marrow cell cultures (Fig. 2). In those cultures, genistein stimulated ALP activity and Ca$^{2+}$ deposition at concentrations between 0.1 and 10 $\mu$M, whereas it inhibited osteogenesis at concentrations of 25 $\mu$M or higher. These data demonstrate that genistein affects osteogenesis of progenitor cells in a biphasic way; namely, it increases osteogenesis at low concentrations and inhibits osteogenesis at high concentrations.
Genistein Binds to and Activates PPARγ

Adipogenesis—Genistein had also a biphasic effect on adipogenesis, which was, however, different to that of E₂ (23). At low concentrations between 0.1 and 1 μM, it decreased adipocyte numbers, while at higher concentrations (>10 μM) it stimulated adipogenesis (Fig. 3A). The effects of genistein on adipogenesis were paralleled by changes in mRNA expression of the adipocyte markers, PPAR-γ2, aP2, and LPL (B), and the number of adipocytes in mouse bone marrow cell cultures (C) were quantified. Each value is the mean ± S.E. of the results from three different wells and is representative of results from at least five different experiments. Significant differences (* < 0.05) were indicated.

ER-dependent and ER-independent Effects of Genistein—Both ER-dependent and ER-independent effects were observed in KS483 cells treated with different concentrations of genistein (Fig. 4). At a concentration of 1 μM, the effects were mediated by ERs because stimulation of ALP activity and inhibition of adipogenesis were both blocked by 1 μM ICI 164,382, a specific antiestrogen. In contrast, at higher concentrations of genistein the effects observed were ER-independent because ICI 164,382 at concentrations from 0.01 to 100 μM did not affect the action of genistein on osteogenesis or adipogenesis. In addition, E₂ (10⁻¹⁰ M to 10⁻⁵ M) did not reverse the effects of genistein at 25 μM on osteogenesis or adipogenesis. These data suggest that the action of genistein at low concentrations is likely ER-mediated, whereas its effects at high concentrations are not ER-mediated.

Activation of PPARγ—We transiently transfected KS483 cells with a luciferase reporter construct containing five copies of a consensus PPRE inserted in front of a TATA box together with expression plasmids encoding human PPAR-γ2. PPRE-luc reporter activity was measured after incubation of transfected cell cultures with different doses of genistein. As shown in Fig 5A, genistein in the micromolar range increased PPRE-luc reporter activity dose-dependently. Furthermore, in the same concentration range, genistein increased PPRE-luc reporter activity in ER-positive and ER-negative breast cancer cell lines, T47D and MDA-MD-231, respectively (not shown). These results were confirmed with three other reporter constructs including the PPARγ response element ACO-luc (25). Thus, genistein transcriptionally activates PPRE-luc reporter activity independently of the cell lines and constructs used.

To determine whether genistein activates PPARγ through direct interaction with this receptor, we performed a membrane-bound PPARγ binding assay. Genistein had a measurable Kᵰ of 5.7 μM (Fig. 5B), which is comparable to that of the known PPARγ ligands (24). We have checked whether genistein bound competitively with [³H]rosiglitazone to the same PPARγ site. Indeed the dissociation constant (Kᵰ) of [³H]rosiglitazone in saturation experiments in the presence of a high dose of genistein was significantly reduced as compared with that in the absence of genistein. The maximal number of sites labeled was not altered. These data demonstrate that both...
Genistein Binds to and Activates PPAR\(\gamma\)

Fig. 5. Genistein is a PPAR\(\gamma\) ligand. KS483 cells were seeded into 24-well plates. After 24 h, they were transiently transfected with a luciferase reporter construct containing five copies of a consensus PPRE inserted in front of TATA box together with expression plasmids encoding human PPAR\(\gamma\)-2. PRE-luc reporter activity was measured after incubation of transfected cell cultures with different doses of genistein for 48 h. Gene reporter assay for PRE-luc shows that genistein stimulates PPAR\(\gamma\) transcriptional activity (A). Binding assays, using a human full-length PPAR\(\gamma\) construct expressed in bacteria, were performed in 96-well plates. The binding assay shows that genistein at the micromolar concentrations binds to PPAR\(\gamma\) (B).

At the micromolar range, genistein binds to and transactivates PPAR\(\gamma\), leading to a decrease of osteogenesis and an increase in adipogenesis. In addition, genistein dose-dependently transactivates ERs, resulting in an up-regulation of osteogenesis and a down-regulation of adipogenesis. Moreover, activation of ERs by genistein could down-regulate PPAR\(\gamma\) transcriptional activity and vice versa. The balance between the activation of ERs and PPAR\(\gamma\) is concentration-related. As a result, the biological effects, i.e. osteogenesis and adipogenesis, vary according to the concentrations of genistein (Fig. 8). Our findings can explain the previously reported diverse actions of genistein in different tissues.

At low concentrations (<1 \(\mu M\)), genistein has ER-dependent effects on osteogenesis and adipogenesis; the effects are similar to those of E2 (23). At high concentrations (>1 \(\mu M\)), however, genistein has antiestrogenic actions, namely, it down-regulates osteogenesis, which is opposite to E2-induced effects. Antiestrogenic effects of genistein have been reported in many cell types and animal models, but the mechanism responsible for this is still not known (1, 2, 4, 26). We show here that the antiestrogenic effects are not due to a decrease of estrogenic activity of genistein. Instead, genistein at micromolar concentrations dose-dependently increased estrogenic transcriptional activity, and the levels were even higher than those induced by E2. These results are in line with reports using different cell lines or assays (6, 27, 28). Moreover, antiestrogenic effects of genistein could not be restored or blocked by E2 or by the antiestrogen compound ICI164,382. Together, our results implicate that antiestrogenic effects of genistein are elicited via pathways other than the ER pathway.

Different from E2, genistein binds to and transactivates PPAR\(\gamma\), leading to adipogenesis. Moreover, activation of PPAR\(\gamma\) may also be due to an inhibition of the MAPK pathway. It is well known that the A/B domain of PPAR\(\gamma\) contains a consensus MAPK site (29–31). Inhibition of PPAR\(\gamma\) phosphorylation by the specific MAPK inhibitor PD98059 stimulates adipogenesis (32). Genistein inhibits p42/44 MAPKs in KS483 cells. 2 It is therefore possible that an inhibition of p42/44 MAPKs contributes to an activation of PPAR\(\gamma\). By using a pure PPAR\(\gamma\) ligand, ciglitazone, we showed that activation of PPAR\(\gamma\) down-regulates osteogenesis in KS483 cells. These results are consistent with observations in MC3T3-E1 cells and in U33 cells (33, 34). It has been shown that PPAR\(\gamma\)-2 plays a dominant role in the determination of the fate of mesenchymal progenitor cells (35). An increase in adipogenesis and a decrease of osteogenesis by genistein at concentrations of 25 \(\mu M\) or higher indicate that PPAR\(\gamma\) actions dominate at higher genistein concentrations.

Genistein concurrently activates two different transcrip-

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2 Z.-C. Dang, V. Audinot, S. E. Papapoulos, J. A. Boutin, and C. W. G. M. Löwik, unpublished observations.
Fig. 6. Antiestrogenic effects of genistein in KS483 cells. KS483 cells were cultured in 12-well plates in the medium containing charcoal-stripped serum and continuously exposed to genistein of 25 μM, E₂ (10⁻⁸ M), and ciglitazone of 25 μM as well as combination of genistein and E₂ for 18 days. ALP activity (A) was measured, which shows that genistein exerts antiestrogenic effects on osteogenesis. KS483 cells containing an integrated ERE-luc reporter gene were exposed to various concentrations of genistein for 48 h. Genistein dose-dependently increased ERE-luc activity (B). When these cells were exposed to E₂ or genistein of 25 μM or in combination of both for 48 h, antiestrogenic effects of genistein were not observed in this gene reporter assay (C). When KS483 cells were cotransfected with ERE-luc reporter gene together with constructs expressing PPARγ2 and exposed to different concentrations of genistein, down-regulation of ERE-luc activity was observed (D). C, control; E, E₂; G, genistein; Ci, ciglitazone.

Fig. 7. Effects of ERα and ERβ on PPARγ transcriptional activity. KS483 cells were seeded into 24-well plates and transiently transfected with a luciferase reporter construct containing five copies of a consensus PPRE inserted in front of TATA box together with expression plasmids encoding human PPARγ2 and in combination with ERα or ERβ. PPRE-luc reporter activity was measured after incubation of transfected cell cultures with different doses of genistein for 48 h. Cotransfection of ERα (A) or ERβ (B) down-regulates PPARγ transcriptional activity. Each value is the mean ± S.E. of the results from three different wells and is representative of results from at least three different experiments. Significant differences (* < 0.05) were indicated.

Fig. 8. Molecular mechanisms of action of genistein. Genistein concurrently activates two different types of transcriptional factors, ERs and PPARγ, which have opposite effects on osteogenesis or adipogenesis. These transcriptional factors influence each other and the balance between activated ERs and PPARγ determines the biological effects of genistein on osteogenesis and adipogenesis.

Our findings provide the molecular basis of the mechanism of action of genistein and may have wide implications. Diverse effects of genistein in different tissues have been explained by the high binding affinity for ERβ because ERβ can act as a dominant negative regulator of estrogenic activity. These dominant negative effects were only observed below the micromolar concentrations of genistein (36). However, the distinct genistein effects in different tissues are often observed at the micromolar concentrations (1, 2, 4, 37). We show that the balance between activated ERs and PPARγ determines the biological effects of genistein, which might explain its diverse biological effects in different organs. Therefore, the biological effects of genistein in certain tissues strongly depend on the concentration of genistein present and the levels of ERs and PPARγ within that particular tissue. There is accumulating evidence that health benefits occur only when phytoestrogens are consumed in sufficient quantities (1, 2, 4). It has been reported that plasma concentration of genistein is relatively low and generally less than 40 nM in humans consuming diets without soy, whereas it can reach 4 μM in the plasma of Japanese who consume high amount of soy products (1, 2, 4). Our findings...
might explain why genistein functions only at a certain level. For example, genistein at the micromolar concentration range inhibits growth of ER-positive breast cancer cells like MCF7 and T47 D as well as ER-negative breast cancer cells like MDA-MB-231 cells (38). Since it is now well established that ligand activation of PPAR\(\gamma\) inhibits cell growth and induces apoptosis in these cancer cells (39–41), it is plausible that only when PPAR\(\gamma\) is activated, genistein at certain levels could inhibit the growth of cancer cells.

Acknowledgments—We are grateful to Drs. E. Kalkhoven, M. G. Parker, J. Auwerx, G. Kuiper, K. van der Lee, M. van Bilsen, K. W. Kinzler and B. Vogelstein for supplying constructs. We thank colleagues from the Endocrinology department for the technical support and Numico Research B. V. for financial support.

REFERENCES

1. Barnes, S. (1998) Proc. Soc. Exp. Biol. Med. 217, 386–392
2. Adlercreutz, H., and Mazur, W. (1997) Ann. Med. 29, 95–120
3. Anderson, J. J. (2001) J. Clin. Endocrinol. Metab. 86, 39–40
4. Setchell, K. D. (1998) Am. J. Clin. Nutr. 68, 1333S–1346S
5. Rumi, M. A., Sato, H., Ishihara, S., Ortega, C., Kadowaki, Y., and Kinoshita, K. (1999) Cell 99, 335–345
6. Morita, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Ohara, T., Ogawa, S., Inoue, S., Muramatsu, M., and Masamune, Y. (2001) Biol. Pharm. Bull. 24, 351–356
7. Barnes, S., Boersma, B., Patel, R., Kirk, M., Darley-Usmar, V. M., Kim, H., and Xu, J. (2000) J. Bone Miner. Res. 15, 217, 4235–4240
8. Lecka-Czernik, B., Moerman, E. J., Grant, D. F., Lehmann, J. M., Manolagas, S. C., and Jilka, R. L. (1999) J. Biol. Chem. 274, 16, 16628–16635
9. Sasaki, T., Fujimoto, Y., Tsuchida, A., Kawasaki, Y., Kuwada, Y., and Chayama, K. (2001) Pathobiology 69, 258–265
10. Rosen, E. D., Hou, C. H., Wang, X., Sakai, S., Freeman, M. W., Gonzalez, F. J., and Spiegelman, B. M. (2002) Genes Dev. 16, 22–26
11. Ren, D., Collingwood, T. N., Rebar, E. J., Woflste, A. P., and Camp, H. S. (2002) Genes Dev. 16, 27–32
12. Nuttall, M. E., and Gimble, J. M. (2000) Bone 27, 177–184
13. Bianco, P., and Gehron, R. P. (2000) J. Clin. Invest. 105, 1663–1668
14. Yamashita, T., Ishii, H., Shimoda, K., Sampath, T. K., Katagiri, T., Wada, M., Osawa, T., and Suda, T. (1999) Genes Dev. 19, 429–436
15. Rumi, M. A., Sato, H., Ishihara, S., Ortega, C., Kadowaki, Y., and Kinoshita, K. (1999) Cell 99, 335–345
16. Calabrese, R. J. (2001) Crit. Rev. Toxicol. 31, 503–515
17. Morita, K., Hirose, T., Kinjo, J., Hirakawa, T., Okawa, M., Ohara, T., Ogawa, S., Inoue, S., Muramatsu, M., and Masamune, Y. (2001) Biol. Pharm. Bull. 24, 351–356
18. De Boer, P., Demare, W., Vanderperre, E., Coeckerman, K., Bossier, P., and Verstraete, W. (2003) Environ. Health Perspect. 110, 691–697
19. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) Science 274, 2100–2103
20. Adams, M., Montague, C. T., Prins, J. B., Holder, J. C., Smith, S. A., Sanders, L., Digby, J. E., Severt, C. P., Lazar, M. A., Chatterjee, V. K., and O’Rahilly, S. (1997) J. Clin. Invest. 100, 3149–3153
Peroxisome Proliferator-activated Receptor \( \gamma \) (PPAR\( \gamma \)) as a Molecular Target for the Soy Phytoestrogen Genistein

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*J. Biol. Chem.* 2003, 278:962-967.
doi: 10.1074/jbc.M209483200 originally published online November 5, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209483200

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