17-Epiestriol, an Estrogen Metabolite, Is More Potent Than Estradiol in Inhibiting Vascular Cell Adhesion Molecule 1 (VCAM-1) mRNA Expression*

Received for publication, August 1, 2002, and in revised form, January 17, 2003
Published, JBC Papers in Press, January 23, 2003, DOI 10.1074/jbc.M207800200

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17-β estradiol (17-β E₂) attenuates the expression of vascular cell adhesion molecule 1 (VCAM-1) in vivo at physiological levels (pg/ml), whereas supraphysiological concentrations of 17-β E₂ (ng/ml) are required in vitro. We assessed whether a metabolite of estrogen, which could only be generated in vivo, might be a more potent inhibitor of VCAM-1 expression and thereby explain this discrepancy. We report here that 17-epiestriol, an estrogen metabolite and a selective estrogen receptor (ER) β agonist, is ~400X more potent than 17-β E₂ in suppressing tumor necrosis factor (TNF-α)-induced VCAM-1 mRNA expression. These actions of 17-epiestriol and genistein were significantly attenuated in the presence of the estrogen receptor antagonist ICI-182780. Other estrogenic compounds such as ethinyl estradiol and estrone did not have any effect on TNFα-induced VCAM-1 expression at the concentrations tested. We further show that, 1) 17-epiestriol induces the expression of endothelial nitric-oxide synthase mRNA and protein, 2) 17-epiestriol prevents TNFα-induced migration of NFκB into the nucleus, 3) N⁰-nitro-L-arginine methyl ester, an inhibitor of NO synthesis, abolishes 17-epiestriol-mediated inhibition of TNFα-induced VCAM-1 expression and migration of NFκB from the cytoplasm to the nucleus. Our results indicate that 17-epiestriol is more potent than 17-β E₂ in suppressing TNFα-induced VCAM-1 expression and that this action is modulated at least in part through NO.

The mechanism by which estrogens attenuate the development of atherosclerosis is not known, although various actions of estrogens have been suggested to mediate this effect (1). We have previously demonstrated that 17-β estradiol (17-β E₂) in vivo inhibits the adhesion of monocytes to endothelial cells of the rabbit aorta (2). We also demonstrated that following a cholesterol-enriched diet to ovariectomized rabbits, expression of VCAM-1 protein was induced in the aorta, and this was attenuated by administration of 17-β E₂ (2). Treatment of cultured rabbit aortic endothelial cells with 17-β E₂ also attenuated the lysophosphatidylcholine-induced expression of VCAM-1 protein. However, the concentrations of 17-β E₂ required to suppress VCAM-1 expression in vivo were in the supraphysiological range when compared with the physiological levels required for in vitro studies. We therefore postulated that one mechanism by which only relatively low concentrations of 17-β E₂ were required for in vivo studies when compared with in vitro studies was that 17-β E₂ might be converted to a more potent metabolite in vivo (2).

Some effects of estrogens are mediated through non-genomic mechanisms, whereas others require transcriptional activation of genes (3). These latter actions usually require that estrogens combine with specific receptors. Two types of estrogen receptors (ER) have been described. They are the “classical” ER, ERα and the “novel” more recently described ERβ (4). Both ERα and ERβ have been detected in endothelial cells and in vascular smooth muscle cells (5, 6). The affinity of various estrogenic compounds for the two ER subtypes markedly differ. 17-β E₂ binds to ERα and ERβ with similar affinity. 17-α ethinyl estradiol (EE), a synthetic estrogen widely used in oral contraceptive formulations, has an ERα-selective agonist potency, whereas 17-α estradiol, an estrogen metabolite, and genistein, a phytoestrogen, at nM concentrations have ERβ-selective agonist potency (7).

The present work was therefore undertaken to test whether certain estrogenic compounds, including an estrogen metabolite, with different agonistic potencies for the two types of estrogen receptors were more potent than 17-β E₂ in suppressing VCAM-1 mRNA expression and the potential mechanism(s) involved. Information obtained from these studies could lead to the development of compounds that attenuate atherogenesis with fewer side effects than those observed with 17-β E₂.

EXPERIMENTAL PROCEDURES

Materials—The culture medium M199, HEPES buffer, and collagenase-type I from porcine skin were obtained from Invitrogen. Gelatin, endothelial-derived growth factor, genistein, TNFα, N⁰-nitro-L-arginine methyl ester (L-NAME), diethyl pyrocarbonate, salmon testes

* This work was supported, in part, by NIA, National Institutes of Health Grant AG-15857. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡† Recipient of the Pfizer/Society for Women’s Health Research Scholars Grant for Faculty Development in Women’s Health.

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1 The abbreviations used are: VCAM, vascular cell adhesion molecule; ER, estrogen receptors; EE, ethinyl estradiol; L-NAME, N⁰-nitro-L-arginine methyl ester; TNF, tumor necrosis factor; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cells; eNOS, endothelial nitric-oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; ELISA, enzyme-linked immunosorbent assay.
DNA, Denhardt’s solution, and SDS were obtained from Sigma; the steroids 17-β E₂, EE, and 17-estriol were also obtained from Sigma with ~99% purity as assessed by thin layer chromatography. Fetal bovine serum (FBS) charcoal/dextran-treated was obtained from HyClone Laboratories. FBS was obtained from Atlas, Fort Collins, CO, and ICI-128780 was obtained from Tocris Cookson Ltd., Ballwin, MO. The human NFκB p65 Nushift kit (2006760) was obtained from Geneka Biotechnology (Montreal, Canada).

**Cell Culture**—For isolation of human umbilical vein endothelial cells (HUVEC), umbilical cords from female fetuses were selected as we observed increased expression of ERα and ERβ mRNA when compared with those obtained from male fetuses. HUVEC were isolated from freshly collected umbilical cords (female fetuses) as previously described (8) and cultured on 0.1% gelatin-coated 75-mm flasks in M199 medium supplemented with 20% FBS, 5 μg/ml endothelial-derived growth factor, 100 μg/ml heparin, 100 units of penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B and 10 mM HEPES buffer, pH = 7.5. Prior to the experiments, the cells were shifted to phenol red-free M199 medium supplemented with 2% charcoal/dextran-treated FBS and the antibiotics mentioned above. Only second or third passage cells were utilized in all the current studies.

**Reverse Transcription-PCR for ERα and ERβ mRNA—**RNA extraction and purification were done using RNasy mini kit as described by the manufacturer’s protocol (Qiagen, Chatsworth, CA). Total RNA (3 μg per sample) was subjected to reverse transcription reaction with 50 units of Moloney murine leukemia virus reverse transcriptase at 42°C for 25 min as previously described (9). The resulting cDNA samples were PCR-amplified using Gene Amp RNA PCR kit (PerkinElmer Life Sciences) according to the manufacturer’s protocol. Oligonucleotide primers were designed for simultaneous PCR amplification (9) of specific DNA fragments contained in both ERα and ERβ using the following set of primers: forward primer sequence, 5’-AAG AGC TGC CAG GCC TGC TG C-3’; reverse primer sequence, 5’-GCC CAG CTG GT C ATG TGA ACC A C-3’.

**Northern Blot Analysis for VCAM-1 and Endothelial Nitric-oxide Synthase (eNOS) mRNA—**10 μg of total RNA was loaded on 1% agarose-formaldehyde gel, electrophoresed, and transferred to Hybond-N (Amersham Biosciences) membrane overnight by capillary action. The RNA was UV-cross-linked using GS Gene Linker (BioRad). VCAM-1 DNA, Denhardt solution, and SDS were obtained from Sigma; the structures of the different estrogens studied are shown in structures panel (A). cDNAs were labeled with [α-32P]dCTP (ICN Biochemicals) using the random priming method. Membranes were prehybridized at 42°C overnight followed by hybridization with respective labeled probes for another 24 h at 42°C. The membranes were washed twice at room temperature with 2 × sodium chloride and sodium citrate (SSC) buffer and 0.5% SDS followed by washing at 65°C for 30 min twice with 0.5 × SSC and 0.1% SDS, and respective bands were quantitated using a Phosphorimager (Molecular Dynamics).

**RESULTS**

**Effect of TNFα on VCAM-1 mRNA Expression and the Modulating Role of ER Agonists—**Second and third passage HUVEC, which were utilized for all our studies, expressed both ERα and ERβ mRNA (data not shown). No basal VCAM-1 mRNA expression was detected in the unstimulated cells or those treated with the different estrogenic compounds alone. The structures of the different estrogens studied are shown in Fig. 1. Preliminary experiments indicated that HUVEC when exposed to 10 ng/ml of TNFα for 4 h led to maximal VCAM-1 mRNA expression. This concentration and time period were utilized for all the experiments.

The effects of preincubation of HUVEC with different concentrations of 17-β E₂, EE (100 and 300 pM, 1, 3, 10, 30, 100, 300 nM), 17-estriol (10, 30, 100, 300 pM), and genistein (1 and 10 μM) on TNFα (10 ng/ml)-induced VCAM-1 mRNA expression are shown in Figs. 2–5. 17-β E₂ at concentrations ranging from 1 to 300 nM attenuated the TNFα-induced VCAM-1 expression, whereas lower concentrations did not have any significant effect when compared with that observed with TNFα alone (Figs. 2 and 3). EE did not have any significant effect on VCAM-1 mRNA expression (Figs. 2 and 3). 17-estriol showed a response that was different from that of 17-β E₂ and EE (Fig. 4). At lower concentrations (30–300 pM), there was significant attenuation of VCAM-1 mRNA expression when compared with that observed with TNFα alone. The maximal attenuation was observed with 100 and 300 pM. The calculated IC50 (mean ± S.D.) for 17-β E₂ and 17-estriol were 16.3 ± 6.8 nM and 36 ± 5.3 pM, respectively. Interestingly, at higher concentrations (1 and 10 nM), the effect of 17-estriol...
was gradually lost, and the VCAM-1 expression started returning to baseline values. However, the magnitude of VCAM-1 expression at 3 nM of 17-epiestriol was still significantly less than that observed with TNFα/H9251 alone. Estriol at similar concentrations to that of 17-epiestriol had no effect (data not shown). Genistein (1 and 10 nM) attenuated VCAM-1 mRNA expression (Fig. 5). The calculated IC50 (mean ± S.D.) for genistein was 7.16 ± 3 nM. The effects of all these estrogen receptor agonists were significantly attenuated in the presence of the ER antagonist ICI-182780 (1 μM), and the results for 17-epiestriol are shown in Fig. 6.

**Effect of 17-Epiestriol on TNFα-induced VCAM-1 Protein Expression**—We next examined the effect of 17-epiestriol on TNFα-induced VCAM-1 protein expression. HUVEC were pre-incubated with different concentrations of 17-epiestriol for 48 h. Six hours after the addition of TNFα, the cells were fixed and assayed for cell-bound VCAM-1 protein by ELISA as described previously (11). A significant reduction in VCAM-1
protein was observed at a 300-pM concentration of 17-epiestriol (Fig. 7). Consistent with the observation related to VCAM-1 mRNA, TNFα-induced VCAM-1 protein expression also showed a biphasic response to 17-epiestriol.

Intermediate Role of Nitric Oxide in the Action of 17-Epiestriol—To determine whether NO played an intermediate role in attenuating TNFα-induced VCAM-1 expression, we first analyzed the effect of 17-epiestriol on eNOS expression. Treatment of HUVEC with 17-epiestriol showed a biphasic response on eNOS protein expression. The maximal increase (68 ± 8%) was observed at 300 pM for both eNOS protein (Fig. 8) and mRNA expression (35%, data not shown). We next assessed the effect of three different concentrations of 17-epiestriol on TNFα-induced VCAM-1 mRNA expression in the absence and presence of L-NAME (3 × 10⁻⁴ M), an inhibitor of NO synthesis. This concentration of L-NAME has been previously demonstrated by us to significantly attenuate basal- and agonist-stimulated NO release by endothelial cells (12). L-NAME either alone or along with TNFα did not affect VCAM-1 expression. 17-epiestriol (100 and 300 pM and 1 nM) attenuated VCAM-1 mRNA expression, and this effect was not observed in the presence of an inhibitor of NO synthesis (Fig. 9).

Effect of 17-Epiestriol on TNFα-induced NFκB Activation—To determine whether 17-epiestriol regulated TNFα-induced VCAM-1 expression by inhibiting NFκB migration to the nucleus, we performed gel-shift assays using oligonucleotides corresponding to the tandem-κB sites on the VCAM-1 promoter. We also assessed whether NO played an intermediate role in this action by 17-epiestriol. 17-epiestriol at concentrations of 100 pM (data not shown) and 300 pM (Fig. 10, lane 5) significantly decreased the intensity of the shifted band produced by nuclear extracts obtained from HUVEC treated with TNFα. However, 10 nM of 17-epiestriol was less effective in decreasing the intensity of the shifted band when compared with 300 pM (Fig. 10, lane 6). Specificity of the complexes was determined by competition with an excess of unlabelled oligonucleotide and with a labeled mutant oligonucleotide when no band was observed (Fig. 10, lane 4). The specificity was further confirmed by supershift analysis (Fig. 10, lane 3) with an affinity-purified polyclonal antiserum to p65. Nuclear translocation of NFκB following treatment with 17-epiestriol in the presence of L-NAME was similar to that observed in the absence of 17-epiestriol and in the presence of L-NAME alone (Fig. 10, lanes 7 and 8). This suggests that the inhibitory effect of 17-epiestriol on the translocation of NFκB to the nucleus following stimulation of HUVEC with TNFα was mainly dependent on NO synthesis.

**DISCUSSION**

The primary objective of this study was to assess whether some selected estrogenic compounds, including a phytoestrogen, a synthetic estrogen, and an estrogen metabolite with different agonistic potencies for ERα and ERβ were more potent than 17β E₂ in attenuating TNFα-induced VCAM-1 expression of HUVEC in vitro. 17β E₂ is the major circulating unconjugated estrogen in premenopausal women. It binds to human ERα and ERβ with approximately similar affinity (7).
Our results indicate that 17-\(\beta\) E\(_2\) did not affect VCAM-1 mRNA expression at lower concentrations (100 and 300 pm), whereas at higher concentrations (1 and 10 nM) decreased VCAM-1 mRNA expression (21). It has recently been demonstrated that a very high concentration of 17-\(\beta\) E\(_2\) is in the pM range, whereas that of 17-epiestriol is considerably lower than that observed with 17-\(\beta\) E\(_2\). This confirmed that the action of the estrogens was receptor-mediated. Further and more specific assessment of the precise ER type involved in mediating the VCAM-1 mRNA expression by estrogens will have to await the discovery of specific ER\(\alpha\) and ER\(\beta\) receptor antagonists. Genistein at nanomolar concentrations has binding affinity to ER\(\beta\), which is similar to that of 17-\(\beta\) E\(_2\), whereas its affinity to ER\(\alpha\) is considerably less (14). Our observation that genistein at nM concentrations (1 and 10 nM) decreased VCAM-1 mRNA expression supports the concept that ER\(\beta\) is most likely involved in the attenuation of VCAM-1 expression in endothelial cells and that ER\(\alpha\) probably has very little role in this phenomenon. At these concentrations, genistein has very little tyrosine kinase inhibitory activity (14) and, hence, the effects are likely due to its action on the ER\(\beta\).

No estrogen-responsive element in the promoter region of the VCAM-1 gene has yet been identified (20), and other indirect mechanisms need to be considered. The VCAM-1 promoter contains consensus binding sites for the nuclear transcription factor NF\(\kappa\)B, the GATA family of transcription factors, and an AP-1 site (15). NF\(\kappa\)B is a proinflammatory transcription factor up-regulating several genes involved in endothelial activation (21). It has recently been demonstrated that a very high concentration of 17-\(\beta\) E\(_2\) (10 \(\mu\)M) suppressed VCAM-1 expression indirectly by inhibiting the nuclear translocation and DNA binding of NF\(\kappa\)B, and this was also associated with a reduction of AP-1 and GATA transcription factors binding to the VCAM-1 promoter (15). These investigators also demonstrated that in human endothelial cells, NF\(\kappa\)B inhibition by E\(_2\) was associated with decreased I\(\kappa\)B-\(\alpha\) degradation.

On this basis we wanted to assess the possibility that estrogens may have increased the synthesis of NO, which in turn...
may have led to the suppression of DNA binding of transcription factors, thereby leading to inhibition of VCAM-1 expression. There is a lot of similarity between the actions of estrogens and NO on various transcription factors that modulate VCAM-1 expression. NO donors inhibited cytokine-induced expression of VCAM-1 and repressed VCAM-1 gene transcription in part by inhibiting nuclear binding protein NF-kB (22, 23). NO also stabilized the NF-kB inhibitor, IxB-a (24), and cellular treatment with NO donor compounds also inhibited AP-1 binding to DNA (25).

We previously demonstrated that estrogens can increase NO production (12), and subsequently others have demonstrated that it can occur through both genomic (26) and non-genomic (27, 28) mechanisms. We therefore decided to assess whether 17-epiestriol at concentrations that this was at least in part due to increased eNOS mRNA. Our results indicated that 17-epiestriol also had a biphasic effect on eNOS protein expression. 17-Epiestriol at a concentration that maximally suppressed TNF-a-induced VCAM-1 mRNA expression also increased eNOS protein and whether this was at least in part due to increased eNOS mRNA. Our results indicated that 17-epiestriol had a biphasic effect on eNOS protein expression. 17-Epiestriol at a concentration that maximally suppressed TNF-a-induced VCAM-1 mRNA expression also increased eNOS protein (Fig. 8) and mRNA, suggesting that NO may play an intermediary role in this action of 17-epiestriol. This was confirmed by observing that the suppression of VCAM-1 mRNA expression by 17-epiestriol was not observed following inhibition of NO synthesis (Fig. 9). Our results also suggest that 17-epiestriol-induced NO production played an intermediate role in inhibition of the nuclear translocation and DNA binding of NF-kB in HUVEC as this inhibitory effect of 17-epiestriol was significantly attenuated in the presence of an inhibitor of NO synthase. Higher concentrations (1 and 10 nM) of 17-epiestriol attenuated the eNOS protein expression when compared with 300 pm, and this may explain the biphasic response of 17-epiestriol on VCAM-1 mRNA and protein expression. Our study did not address the precise molecular mechanism(s) by which NO production stimulated by 17-epiestriol. This was confirmed by observing that the suppression of VCAM-1 and repressed VCAM-1 gene transcription of 17-epiestriol in modulating VCAM-1 gene and protein expression indicate that estrogens may have paradoxical effects in inflammatory processes. This may be one potential explanation for the controversy in relation to the effects of estrogen replacement therapy on cardiovascular morbidity (17).

Acknowledgments—We thank Sripura Mukhopadhyay, Julie Gage, Janis Cuevas, and Svetlana Arutyunova for technical assistance.

REFERENCES

1. Nathan, L., and Chaudhuri, G. (1997) Annu. Rev. Pharmacol. Toxicol. 37, 437–455
2. Nathan, L., Pervin, S., Singh, R., Rosenfeld, M., and Chaudhuri, G. (1999) Circ. Res. 85, 377–385
3. Haynes, M. P., Russell, K. S., and Bender, J. R. (2000) J. Nucl. Cardiol. 7, 560–568
4. Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J.,Nilsson, S., Gustafsson, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5925–5930
5. Register, T. C., and Adams, M. B. (1988) J. Steroid Biochem. Mol. Biol. 34, 187–191
6. Lindner, V., Kim, S. K., Karas, R. H., Kuiper, G. G., Gustafsson, J. A., and Mendelec, M. E. (1998) Circ. Res. 83, 224–229
7. Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J., and Nilsson, S. (1998) Mol. Pharmacol. 54, 105–112
8. Caulin-Glaser, T., Watson, C. A., Pardi, R., and Bender, J. R. (1996) J. Clin. Invest. 98, 36–42
9. Hodges, Y. K., Tung, L., Yan, X. D., Graham, J. D., Horwitz, K. B., and Horwitz, L. D. (2000) Circulation 101, 1792–1798
10. Singh, R., Pervin, S., Karim, A., Cedherbaum, S., and Chaudhuri, G. (2000) Cancer Res. 60, 3305–3312
11. Mukherjee, T. K., Dinh, H., Chaudhuri, G., and Nathan, L. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4055–4060
12. Hayashi, T., Fukuto, J. M., Ignarro, L. J., and Chaudhuri, G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11209–11263
13. Yeh, N. N., Venugopalan, M., Harlukar, S., and Glassbrook, A. (1996) Science 273, 1223–1225
14. Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., and Gustafsson, J. A. (1998) Endocrinology 139, 4525–4533
15. Simoncini, T., Maffei, S., Basta, G., Barsacchi, G., Genazzani, A. R., Liao, J. K., and De Caterina, R. (2000) Circ. Res. 87, 19–25
16. Nakai, K., Roh, C., Hotta, K., Hoh, T., Yoshizumi, M., and Hiramori, K. (1994) Life Sci. 54, PL212–PL227
17. Writing Group for the Women's Health Initiative Investigators (2002) JAMA 288, 321–333
18. Cid, M. C., Kleinman, H. K., Grant, D. S., Schnaper, H. W., Fauci, A. S., and Hoffman, G. S. (1994) J. Clin. Invest. 93, 17–25
19. Aziz, K. E., and Wakefield, D. A. (1996) Cell. Immunol. 167, 79–85
20. Iademarco, M. F., McQuillan, J. J., Rosen, G. D., and Dean, D. C. (1992) J. Biol. Chem. 267, 16323–16329
21. De Caterina, R., and Gimbrone, M. A., Jr. (1995) in n-3 Fatty acids Prevention and Treatment in Vascular Disease (Kristensen, S. D., Schmidt, E. B., and Gimbrone, M. A., Jr., editors) pp. 9–24, Springer Verlag, London
22. De Caterina, R., Libby, P., Peng, H. B., Thannickal, V. J., Rajavashisth, T. B., Gimbrone, M. A., Jr., Shin, W. S., and Liao, J. K. (1996) J. Clin. Invest. 96, 60–68
23. Spiecker, M., Darius, H., Kaboth, K., Ruhrmann, F., and Liao, J. K. (1998) Leukoc. Biol. 63, 732–739
24. Katsuyama, K., Shichiri, M., Marumo, F., and Hrita, Y. (1998) Arterioscler. Thromb. Vasc. Biol. 18, 1796–1802
25. Takahashi, A., Sano, K., Oh, E., Tsuchiya, T., and Tsuda, M. (1994) FERS Lett. 351, 123–127
26. Weiner, C. P., Linares, I., Blyas, S. A., Knudtson, R. G., and Charles, I. G., Noronha, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5312–5316
27. Caulin-Glaser, T., Garcia-Cardenas, G., Sarrel, S., Sessac, W. C., and Bender, J. R. (1997) Circ. Res. 81, 885–892
28. Chen, Z., Yuhanna, I. S., Galcheva-Gargova, Z., Karas, R. H., Mendelsohn,
M. E., and Shaul, P. W. (1999) *J. Clin. Invest.* **103**, 401–406, 1363

29. Paech, K., Webb, P., Kuiper, G. G. J. M., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997) *Science* **277**, 1508–1510

30. Iafrati, M. D., Karas, R. H., Aronovitz, M., Kim, S., Sullivan, T. R., Jr., Lubahn, D. B., O’Donnell, T. F., Jr., Korach, K. S., and Mendelsohn, M. E. (1997) *Nat. Med.* **3**, 545–548

31. Karas, R. H., Hodgson, J. B., Kwoun, M., Krege, J. H., Aronovitz, M., Mackey, W., Gustafsson, J. A., Korach, K. S., Smithies, O., and Mendelsohn, M. E. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 15133–15136

32. Makela, S., Savolainen, H., Aavik, E., Myllarniemi, M., Strauss, L., Taskinen, E., Gustafsson, J. A., and Hayry, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 7077–7082