Effect of epidermal growth factor and prostaglandin on the expression of aromatase (CYP19) in human adrenocortical carcinoma cell line NCI-H295R cells

Masatada Watanabe, Mariko Noda and Shizuo Nakajin

Department of Biochemistry, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan

(Requests for offprints should be addressed to M Watanabe; Email: masatada@hoshi.ac.jp)

Abstract

We investigated the effects of epidermal growth factor (EGF) and prostaglandins (PG) on the expression of aromatase (CYP19) in human adrenocortical carcinoma cell line NCI-H295R cells. EGF significantly increased aromatase activity and CYP19 gene transcript in NCI-H295R cells. Exon PII was selected from among several tissue-specific exon I regions. Promoter II that abuts on exon PII was activated by EGF. PGE2 also significantly increased aromatase activity, CYP19 gene transcript, and promoter II activity. The results of experiments using protein kinase (PK) inhibitors suggest that the cAMP–PKA signaling pathway is involved in the up-regulation of aromatase expression by EGF. PGE2 activated promoter II activity in 4 h, while 12 h was required for its activation by EGF. In addition, PGE2 was secreted from NCI-H295R cells in response to EGF. Selective agonists for prostaglandin receptors EP1 and EP2 significantly increased aromatase activity, which was decreased by the corresponding antagonists. Finally, antagonists for EP1 and EP2 inhibited the up-regulation of aromatase expression following EGF. These results suggest that PGE2 secondarily acts as an autocrine signal in the up-regulation of aromatase expression by EGF in NCI-H295R cells.


Introduction

There have been reports indicating that tumors which secrete abnormally high levels of estrogen cause gynecomastia in boys and men and precocious puberty in girls. In these cases, cytochrome P450 aromatase (CYP19), the key enzyme of estrogen biosynthesis, is highly expressed (Young et al. 1996, Watanabe et al. 2000, Phornphutkul et al. 2001). In another case, a mutation in the promoter region of the CYP19 gene that codes for aromatase caused excess expression of aromatase and progression of gynecomastia (Shozu et al. 2003).

CYP19 gene is composed of multiple untranslated exon I and coding regions (exon II to exon X) (Bulun et al. 2004). A special feature of the CYP19 gene is its multiple exon I. At least ten exon I regions are selected in a tissue-specific fashion. After alternative splicing, mRNA with a tissue-specific 5’-untranslated region is formed. In this tissue-specific selection of exon I, promoter regions that abut on the 5’-end of each exon I are activated by a distinct signal pathway. For example, promoter II and promoter I.3 that abut on the 5’-end of exon PII and exon I.3 respectively, are activated by the signaling pathway that includes the elevation of intracellular cAMP concentration. On the other hand, promoter I.4 that abuts on the 5’-end of exon I.4 is activated by glucocorticoid.

We found previously that forskolin largely up-regulates aromatase expression in NCI-H295R cells, a human adrenocortical carcinoma cell line (Watanabe & Nakajin 2004). Promoter I.3 and promoter II are activated in NCI-H295R cells. From these findings, we were interested in identifying physiological factors that induce aromatase expression in NCI-H295R cells in order to clarify the mechanism of aromatase expression in adrenal tumors.

Epidermal growth factor (EGF) increases aromatase activity and expression in MCF-7 and adipose stromal cells and induces expression of cyclooxygenase 2 (COX-2) in adipose stromal cells (Richards et al. 2002). In breast tumors, prostaglandin (PG) E2 increases intracellular cAMP levels and stimulates estrogen biosynthesis (Zhao et al. 1996); furthermore, it up-regulates aromatase activity and expression in adipose stromal cells (Richards & Brueggemeier 2003). EGF affects the expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) type II and CYP17 in NCI-H295R cells (Doi et al. 2001). Also in NCI-H295R cells, up-regulation of aromatase expression by PGE2 has been reported (Heneweer et al. 2004). However, the mechanisms of the effects of EGF and PGE2 on aromatase expression in NCI-H295R cells have not been examined in detail at the molecular biological level. Therefore, we conducted detailed studies on the effects...
of EGF and prostaglandins on aromatase expression in NCI-H295R cells.

Materials and Methods

Materials

EGF was purchased from Cosmo Bio Co. Ltd (Tokyo, Japan). EGF was dissolved in 10 mM acetic acid containing 0.1% BSA. Prostaglandin A1 (PGA1) and PGF2α were obtained from Cayman Chemical (Ann Arbor, MI, USA). PGE1 and PGE2 were purchased from Sigma Aldrich Japan (Tokyo, Japan). Prostaglandins were dissolved in ethanol. H-89 was purchased from Seikagaku Corporation (Tokyo, Japan) and PD98059 and KN-93 from Cosmo Bio Co. Ltd. Protein kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO). Butaprost, sulprostone, AH6809 and SC-19220 were obtained from Cayman Chemical. ONO-DI-004 and ONO-AE1–259–01 (Suzawa et al. 2000, Narumiya & FitzGerald 2001) were generous gifts provided by Ono Pharmaceutical Co. Ltd (Osaka, Japan). Butaprost, ONO-DI-004 and ONO-AE1–259–01 were dissolved in ethanol. Sulprostone, AH6809 and SC-19220 were dissolved in DMSO. The concentration of each solvent was 0.1% (v/v). D-MEM/F-12, D-MEM/F-12 without phenol red and a mixture of penicillin (5000 U/ml) and streptomycin (5000 µg/ml) were purchased from Invitrogen Japan K.K. (Tokyo, Japan). ITS plus was purchased from Nippon Becton Dickinson Company, Ltd (Tokyo, Japan). Nu-Serum I was purchased from Cosmo Bio Co. Ltd and fetal calf serum (FCS) was from Sanko Junyaku (Tokyo, Japan).

Cells

NCI-H295R human adrenocortical carcinoma cells were purchased from the American Type Culture Collection (Manassas, VA, USA). NCI-H295R cells were cultured in D-MEM/F-12 supplemented with 50 µg/ml penicillin, 50 µg/ml streptomycin, 1% (v/v) ITS plus and 2.5% (v/v) Nu-Serum I. The human granulosa-like cell line KGN (Nishi et al. 2001) was obtained from Riken Cell Bank (Tsukuba, Japan). KGN cells were cultured in D-MEM/F-12 medium supplemented with penicillin (50 µg/ml), streptomycin (50 µg/ml) and FCS (10% v/v). Cells were maintained as monolayer cultures in 10-cm dishes at 37 °C in an atmosphere of 5% CO2-95% air.

Aromatase assay

NCI-H295R cells were seeded at a density of 5·0 × 10^5 cells/well on 24-well plates. After culturing for 24 h, the medium was replaced with the treatment medium (D-MEM/F-12 without phenol red with 1% (v/v) ITS plus, penicillin (50 U/ml) and streptomycin (50 µg/ml)). After incubation for 24 h (serum starvation), the cells were treated with treatment medium that contained peptide or chemical compounds. After incubation, aromatase activity in the NCI-H295R cells was measured by the tritiated water method described previously (Watanabe & Nakajin 2004).

RT-PCR

After serum starvation, the NCI-H295R cells were treated with EGF for 24 h or PGE2 for 4 h. Then, total RNA was extracted using ISOGEN (Nippongene, Toyama, Japan). Human placenta ribonuclease inhibitor (Takara Shuzo, Shiga, Japan) and AMV reverse transcriptase (Promega) according to the manufacturers’ instructions. The RT-PCR of the gene transcripts of EGF receptor (EGFR), CYP19 (coding region (exon IX-X) and exon PII-including 5′ region), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and prostaglandin receptors (EP1, EP2, EP3 and EP4) were based on published data (Moroni et al. 2001, Schlotzer-Schrehardt et al. 2002, Iwasaki et al. 2003, Richards & Brueggemeier 2003, Watanabe & Nakajin 2004). The PCR products were visualized with ethidium bromide after electrophoresis on 1.5% agarose gel, and quantified by calculating the intensity of the band of the PCR product of CYP19 (coding region (exon IX-X) and exon PII-including 5′ region) relative to the intensity of the band of the PCR product of GAPDH.

Transfection and luciferase assay

NCI-H295R cells were seeded at a density of 5·0 × 10^5 cells/well on 24-well plates. After culturing for 24 h, firefly luciferase reporter vectors harboring various lengths of promoter L3/II or empty firefly luciferase vector (pGL3-Basic) and sea pansy luciferase internal control vector (phRL-TK) were transfected using Fugene 6 transfection reagent (Roche Diagnostics K.K., Tokyo, Japan). After 24-h transfection, the cells were serum starved for 4 h. The cells were then treated with peptides and chemical compounds. After treatment, the cells were lysed and luciferase activities in the lysate were measured using a Dual-Luciferase reporter assay (Promega) and a Sirius luminometer (Berthold Detection Systems, Pforzheim, Germany).

Enzyme immunoassay of PGE2

The concentration of PGE2 in the conditioned media of NCI-H295R cells that were treated with EGF for
Statistical analysis
The statistical significance of experimental data was determined by one-way ANOVA followed by the Dunnett, Tukey, or Bonferroni/Dunn tests for multiple comparisons.

Results

Effect of EGF on aromatase expression in NCI-H295R cells
Before evaluation of the effect of EGF, we tested the expression of EGFR gene transcript in NCI-H295R cells by RT-PCR. As shown in Fig. 1A, the EGFR gene transcript was expressed in NCI-H295R cells. We then tested the effect of EGF on aromatase expression in NCI-H295R cells. As shown in Fig. 1B, aromatase activity in the cells was significantly increased by EGF in a concentration-dependent fashion. We also tested the effect of EGF on the expression of the CYP19 gene. As shown in Fig. 2A and B, one of the CYP19 gene transcripts corresponding to the coding region and the other gene transcript which includes exon PI was both up-regulated by EGF in a concentration-dependent manner. These results suggest that EGF up-regulates aromatase expression at the level of CYP19 gene expression in NCI-H295R cells.

Effects of prostaglandins on aromatase expression in NCI-H295R cells
We tested the effects of prostaglandins (prostaglandin A1 (PGA1), PGB2, PGD2, PGE1, PGE2 and PGF2α) on aromatase expression in NCI-H295R cells. As shown in Fig. 6, all the prostaglandins except PGF2α increased aromatase activity significantly. The increases in aromatase activity by PGE1 and PGE 2 were particularly large.

Effect of EGF on promoter I.3/II activity of the CYP19 gene
There are two cAMP-responsive promoter regions upstream of exon PI in the CYP19 gene. Promoter II abuts on the 5′-end of exon PI and this promoter includes the sequence of exon I.3. Promoter I.3 abuts on exon I.3. To clarify the mechanism of CYP19 gene expression, we transfected the firefly luciferase reporter constructs that harbor several 5′-deleted promoter I.3/II sequences as shown in Fig. 3. The results in Fig. 4 show that the reporter activity (promoter activity) of −717/+23 Luc was activated significantly and in a concentration-dependent fashion by treatment with EGF for 12 h. This result suggests that activation of the promoter region that abuts on the 5′-end of exon PI is involved in the up-regulation of aromatase expression by EGF. To clarify the sequence that is important for the activation of promoter I.3/II, we transfected the deletion constructs into NCI-H295R cells, treated the cells with 100 ng/ml EGF, and then measured promoter activity. As shown in Fig. 5, −717/+23 Luc and −278/+23 Luc had the same promoter activity. However, the promoter activity of −227/+23 Luc, which has a deletion sequence that includes TATA of promoter I.3, was less than half that of −278/+23 Luc. This result suggests that the 51 base pair sequence that includes TATA of promoter I.3 is important for the promoter activity. In addition, −198/+23 Luc was still activated significantly by EGF, while −119/+23 Luc was not significantly activated. This result suggests that the 79 base pair sequence between −198 and −119 in promoter II is critical for the activation of promoter I.3/II by EGF.
Based on these findings, we concentrated our efforts on the effect of PGE₂. As shown in Fig. 7A, four subtypes of prostaglandin receptors (EP₁, EP₂, EP₃, and EP₄) were expressed in NCI-H295R cells. Furthermore, as shown in Fig. 7B, aromatase activity in NCI-H295R cells was increased by PGE₂ significantly and in a concentration-dependent manner. As shown in Fig. 8A and B, CYP19 gene transcripts which include the coding region and exon PII sequences were both up-regulated in response to PGE₂ in a concentration-dependent fashion. These results suggest that PGE₂ up-regulates aromatase expression at the level of CYP19 gene expression.

Effect of PGE₂ on the promoter I.3/II activity of CYP19 gene

As was the case for EGF, we tested the effect of PGE₂ on promoter I.3 and promoter II. As shown in Fig. 9, the promoter activity of −717/+23 Luc was activated significantly and in a concentration-dependent manner. PGE₂ activated promoter activity in 4 h whereas upregulation with EGF required 12 h. We also tested the effect of PGE₂ on a deletion construct of promoter I.3/II. As shown in Fig. 10, reporter activity decreased as the promoter length was shortened. The deletion construct −198/+23 Luc was still activated by PGE₂ significantly, while −119/+23 Luc was not activated significantly. This result suggests that the 79 base pair sequence between −198 and −119 in promoter II includes an important sequence, as was the case for EGF.

Effects of EP receptor-specific agonists and an antagonist

To elucidate which PGE₂ receptor (EP₁, EP₂, and EP₃) is involved in the induction of aromatase expression,
we compared aromatase activity in the presence of some agonists and an antagonist. As shown in Fig. 11, aromatase activity was induced by ONO-DI-004 (selective agonist for EP₁), ONO-AE1–259–01 (selective agonist for EP₂) and butaprost (selective agonist for EP₂). These inductions were completely inhibited by AH6809 (antagonist for EP₁ and EP₂). It was not possible to obtain specific agonists and antagonists for EP₄. These data suggest that EP₁ and EP₂ are involved in the induction of aromatase in NCI-H295R cells. The fact that there was no induction by sulprostone (selective agonist for EP₃) suggests that EP₃ is not involved in the induction.

**Effects of protein kinase inhibitors**

We tested the effects of several protein kinase (PK) inhibitors to elucidate the intracellular signaling pathways that up-regulate aromatase expression. As shown in Fig. 12, up-regulation of aromatase activity by EGF was significantly inhibited to varying degrees by PD98059 (inhibitor of MAP kinase kinase), KN-93 (inhibitor of calcium–calmodulin kinase II) and H-89 (inhibitor of PKA). Aromatase activity was down-regulated to 35.3% of the value with EGF alone by PD98059. Aromatase activity was below the control level in the presence of KN-93. Interestingly, aromatase activity was down-regulated to 21.1% by H-89 (Fig. 12A). This result suggests that the cAMP–PKA pathway is involved in the up-regulation of aromatase in response to EGF. The aromatase activity in response to PGE₂ was down-regulated to 65.9% of the value with PGE₂ alone by

**Figure 3** Position of multiple exon I and putative transcription factor binding sites around exon II of the human CYP19 gene. The positions of multiple exon I (top) and transcription factor binding sites (middle: CREaro, TATA, CLS, SF-1, S₁) in the partial genomic sequence of the CYP19 gene are indicated. The positions of genomic sequences inserted into firefly luciferase reporter vector (bottom) are also indicated.

**Figure 4** EGF activates promoter I.3/II region in NCI-H295R cells. −717/+23 Luc firefly luciferase reporter vector or empty firefly luciferase vector (Basic) was transfected into NCI-H295R cells with a sea pansy luciferase internal control vector (phRL-TK) using Fugene 6 transfection reagent. After transfection for 24 h and then serum starvation for 4 h, cells were treated with EGF for 12 h. Then, luciferase activity in the cell lysate was measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Dunnett’s multiple comparison test. Asterisks denote significant differences compared with the vehicle control (0 ng/ml); *P<0.05.

**Figure 5** Effect of EGF on the activity of the deletion series of promoter I.3/II sequence. 5’-deleted series of firefly luciferase reporter vector that were shown in Fig. 3 or empty firefly luciferase vector (Basic) were transfected into NCI-H295R cells with a sea pansy luciferase internal control vector (phRL-TK) using Fugene 6 transfection reagent. After transfection for 24 h and then serum starvation for 4 h, cells were treated with EGF for 12 h. Then, luciferase activity in the cell lysate was measured. Each column represents the mean with S.E.M. (n=3). The differences between means were analyzed by one-way ANOVA followed by Bonferroni/Dunn’s multiple comparison test. Asterisks denote significant differences compared with the vehicle control (−); *P<0.05.
PD98059 and to 42.4% by KN-93. H-89 almost completely diminished aromatase activity (Fig. 12B). These results suggest that the cAMP–PKA pathway is the main signaling pathway in the up-regulation of aromatase in response to PGE2. These results also suggest that the cAMP–PKA pathway evoked in response to PGE2 would be involved in the up-regulation of aromatase expression in response to EGF in NCI-H295R cells.

**EGF induces PGE2 secretion from NCI-H295R cells**

The results of the experiments using several protein kinase inhibitors suggest that the PGE2–cAMP–PKA pathway may be involved in the up-regulation of aromatase expression in response to EGF. PGE2 secreted from NCI-H295R cells in response to EGF may stimulate the cells in an autocrine fashion. Based on this, we attempted to determine whether PGE2 is secreted from NCI-H295R cells in response to EGF. As shown in Fig. 13, the secretion of PGE2 in conditioned media increased in a concentration-dependent manner in response to EGF, but the secretion of PGD2 and PGE1 did not respond to EGF (data not shown). This result suggests that PGE2 acts partly as a secondary factor in the up-regulation of aromatase expression by EGF.

**EP receptor antagonists down-regulate aromatase activity which is induced by EGF**

To confirm that PGE2 is the secondary factor in up-regulation of aromatase expression by EGF, we tested if EP receptor antagonists inhibit the induction of aromatase expression by EGF. As shown in Fig. 14, induction of aromatase activity with EGF was inhibited with AH6809 (antagonist for EP1 and EP3) and SC-19220 (selective antagonist for EP4). This result suggests that the PGE2 signaling pathway (at least EP1 and EP3) is involved in the induction of aromatase expression in NCI-H295R cells by EGF.

**Discussion**

Aromatase is not expressed in normal adrenal cortex, but it is expressed after carcinogenesis. This fact suggests that there would be common molecular mechanisms between carcinogenesis and aromatase expression in adrenocortical carcinoma. Therefore, investigation of the mechanism of CYP19 gene expression in estrogen-secreting adrenocortical carcinomas may supply helpful data for determining the mechanism of carcinogenesis in the adrenal cortex. An adrenocortical carcinoma itself or its primary culture would be suitable tools for this kind of investigation.
However, because of the shortage of estrogen-secreting adrenocortical carcinomas and the difficulty associated with molecular investigation (e.g. transfection experiments), these tools are not suitable for studying the molecular biological mechanism. On the other hand, a cell line that is established from an adrenal tumor is easy to culture and stock, and the cell line is easily transfected. Taking into consideration these features, the human adrenocortical carcinoma cell line NCI-H295R can be a useful model of estrogen-secreting adrenocortical carcinoma.

The effects of EGF and PGE₂ on aromatase activity and expression have been well studied in human adipose stromal cells (Richards et al. 2002, Richards & Brueggemeier 2003). The up-regulation of aromatase activity and gene transcript with PGE₂ in H295R cells has been reported (Heneweer et al. 2004).

In this study, we investigated the molecular biological mechanism of the up-regulation of aromatase expression in NCI-H295R cells in response to EGF and PGE₂. Both EGF and PGE₂ up-regulated aromatase expression at the level of CYP19 gene transcription. The results of transfection experiments using a deletion construct of promoter I.3/II suggest the existence of an important sequence in the 79 base pairs between p119 and p119 in promoter II. The binding site for steroidogenic factor 1 (SF-1) is in this sequence. Since the mutation in SF-1 site abolished the response of promoter II to forskolin (Watanabe & Nakajin 2004), SF-1 or another transcription factor that binds to this site must have an important role.

EGF, a ubiquitous hormone, would exert its effect on the adrenal cortex in an endocrine fashion. Furthermore, transforming growth factor-α, which has a similar structure to EGF and is able to bind to EGFR, is expressed in adrenal cortex (Sasano et al. 1994). Based on these observations, downstream of the EGFR signaling pathway could be evoked in adrenocortical carcinomas.
Prostaglandins can be synthesized in an adrenocortical carcinoma, and they can work in an autocrine or paracrine fashion. In rabbit chondrocyte and human squamous carcinoma cell lines, EGF induced the secretion of PGE2 via up-regulation of the activities of phospholipase A2 (PLA2) and COX-2 (Sato et al. 1997, Huh et al. 2003). This may suggest that PGE2 acts as a secondary factor to EGF in the up-regulation of aromatase expression. Therefore, we checked whether PGE2 was secreted from NCI-H295R cells in response to EGF. In this study, NCI-H295R cells secreted PGE2 in response to EGF (Fig. 13), and PGE2 increased aromatase activity to a greater extent than other prostaglandins (Fig. 6). The inhibition of EGF-induced aromatase expression with PGE2 receptor antagonists confirmed that PGE2 is the secondary factor of aromatase expression with EGF (Fig. 14). PGE1 also increased aromatase activity to a degree similar to that of PGE2, but EGF could not stimulate NCI-H295R cells to secrete a sufficient concentration of PGE1 (data not shown) to increase aromatase activity. These results suggest that several prostaglandins are secreted in response to EGF, and that these prostaglandins evoke some intracellular signaling pathways. According to the experiments using several protein kinase inhibitors (Fig. 12), the intracellular signaling pathways that include MAP kinase, and calcium-calmodulin kinase are important for up-regulation of aromatase by EGF. In response to EGF, EGF receptors (receptor-type tyrosine kinase) activate the MAP kinase pathway through phosphorylation of Ras protein. It is also well known that EGF receptors increase the intracellular calcium concentration. Therefore, it would be reasonable to conclude that inhibition of MAP kinase and calcium-calmodulin kinase II down-regulate aromatase expression in NCI-H295R cells. Interestingly, a PKA inhibitor (H-89) down-regulated aromatase activity. This result suggests that the cAMP–PKA pathway is involved in the up-regulation of aromatase expression in response to EGF.
by EGF. Probably a second factor induced by EGF would increase the intracellular cAMP concentration. PGE₂ is known to increase the intracellular cAMP concentration. In addition, PGE₂ is secreted from NCI-H295R cells in response to EGF (Fig. 13). Furthermore, the induction of aromatase expression with PGE₂ might depend mainly on the PKA pathway (Fig. 12B). Thus, PGE₂ would be a second factor involved in the up-regulation of aromatase expression by EGF. Up-regulation of aromatase expression with PGE₂ appears to depend partially on MAP kinase kinase and calcium-calmodulin kinase II (Fig. 12B). These phenomena could be explained by the existence of EP₁, which has some relationship with the signaling pathways involving calcium and the transactivation of EGF receptors with PGE₂ (Pai et al. 2002). This signaling pathway would act synergistically with other signals in response to EGF to up-regulate aromatase expression.

The result of the experiments using several agonists and antagonists of EP receptors suggest that EP₁ and EP₂ are involved in the up-regulation of aromatase expression but that EP₃ is not involved.

In the present study, we showed that EGF and prostaglandins up-regulate aromatase expression in NCI-H295R cells. The autocrine and paracrine system of aromatase expression...
up-regulation in adrenal tumors is similar to the system in human adipose stromal cells surrounding breast cancer (Richards et al. 2002, Richards & Brueggemeier 2003). In addition, we investigated the molecular mechanism of the up-regulation of aromatase expression by EGF and PGE$_2$ using promoter analysis and protein kinase inhibitors. However, the up-regulated aromatase activity is lower than the aromatase activity in adrenocortical carcinoma (Watanabe et al. 2000). Therefore, multiple factors, not only EGF and prostaglandins, would synergistically up-regulate aromatase expression at a high level. The search for such factors would help to clarify the mechanism of high level aromatase expression in adrenocortical carcinomas. Finally, it is hoped this search will assist in the development of drugs that down-regulate aromatase expression specifically in adrenocortical carcinomas.

Acknowledgements

We thank Ono Pharmaceutical Co. Ltd for providing ONO-DI-004 and ONO-AE1–259–01. This work was supported, in part, by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan and by Health Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 23 September 2005
Accepted 13 October 2005
Made available online as an Accepted Preprint 9 November 2005

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