Insulin Attenuated Estrogen Receptor in Neutrophil Dwindled Synthesis of Maspin in Breast Cancer

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Abstract ***Purpose***: The binding of either estrogen or insulin to their specific receptors on neutrophils has been reported to stimulate nitric oxide (NO) induced maspin synthesis in these cells. Experiments were carried out to determine the role of estrogen receptor interaction in the nitric oxide induced maspin synthesis in neutrophils that was preincubated with insulin. ***Methods***: Estrogen receptor positive (ER+), estrogen receptor negative (ER–) neutrophils were isolated from the blood cancer subjects. Maspin was determined by enzyme linked immunosorbent assay after in vitro translation of maspin mRNA. NO was determined by methemoglobin method. ***Results***: Immunohistochemical studies of estrogen receptor (ER) demonstrated the presence of both ERα and ERβ subtypes in the normal peripheral neutrophils and less in number in ER+ breast cancer neutrophils. In contrast, ER– breast cancer neutrophils lacked the ERα and ERβ subtypes, suggesting pathophysiological defects in the synthesis of ER (α and β) proteins in peripheral ER– neutrophils. ***Conclusion***: These results suggested that insulin dwindled maspin synthesis in normal and in breast cancer neutrophils by decreasing the estrogen receptor number in both cases.

Keywords Breast cancer · Estrogen · Estrogen receptor · Insulin · Maspin · Nitric oxide · Neutrophils

1 Introduction

Breast cancer is most frequently encountered in women and is an estrogen dependant condition (Schneider and Jackisch 1998). Estrogen plays an important role in the synthesis of an anti-breast cancer protein, maspin (Mammary Serine proteinase inhibitor, Mr. 42 kDa) which is abundantly expressed in the normal mammary epithelial cells and neutrophils, is reported to inhibit malignant breast cell invasion, angiogenesis, metastasis and promote apoptosis (Zou et al. 1994; Hojo et al. 2001; Liu et al. 1999a).

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The expression of the effect of estrogen is reported to be mediated through the binding of the hormone to the nuclear receptors, and a DNA binding domain which recognizes a sequence of DNA known as hormone responsive elements (HRE) (Beato and Klug 2000).

The estrogen receptor status has prognostic significance (Maehle et al. 2009). The breast cancer tumors that had no receptors of estrogen are reported to results in worse prognostic outcome than those in cases where the receptor of this hormone was present (Rosa et al. 2008). On the basis of presence of estrogen receptor in the lesion, the breast cancer patients are classified into two groups: (1) estrogen receptor positive (ER+) and (2) estrogen receptor negative (ER−). The presence of estrogen receptor (ER+) is a better prognostic indicator for the breast cancers. In contrast, the estrogen receptor negative (ER−) tumors have been reported to have a poorer prognosis than that of the estrogen receptor positive (ER+) tumors (Rosa et al. 2008). It is seen that in patients with ER+ tumors the occurrence of metastases is 3–6 times less probable than in patients with ER− tumors. The clinical data indicated that the ER− breast cancers are less sensitive to therapy than those with ER+ tumors, and ER− patients had a shorter disease free interval than those of ER+ patients (Gelbfish et al. 1988).

Taking these reports together it could be suggested that estrogen plays important role in both the control of breast cancer and in the better prognostic outcome of the malignancy (Schneider and Jackisch 1998).

As reported earlier, estrogen and its receptors have critical role in the development and in the prognostic outcome of the human breast cancer. The role of estrogen in relation to the receptor ligand interaction in the synthesis of maspin is also having paramount importance. The interrelation between the estrogen receptors function and maspin synthesis through NO production in the control of human breast cancer is already established (Ganguly Bhattacharjee et al. 2012). The physiologic events are the consequent effects of binding of one ligand to its own receptors, which influences the binding and the effect of a second ligand to its own receptors and is known as “cross talk” between the receptors (Basrawala et al. 2006; Kahn and Sinha 1992; Freychet et al. 1971; Dutta-Roy et al. 1991; Kahn et al. 1993). We report here that estrogen and insulin were capable of stimulating maspin synthesis through the production of NO in neutrophils. The results of the investigation suggest the existence of effect of insulin on the function of estrogen in neutrophils, in the context of NO induced maspin synthesis. The possible pathological implication of the crosstalk (Ganguly Bhattacharjee et al. 2012; Kahn and Sinha 1990a; Girish et al. 2006) between the receptors of the steroid and the anti-diabetic hormone in the synthesis of the anti-breast cancer protein in human breast cancer are presented herein.

2 Materials and Methods

Ethical Clearance: The protocol used in the study was approved by the Internal Review Board, Sinha Institute of Medical Science and Technology. Appropriate permission was also obtained from the I.R.B. for the use of rabbits in the studies.

Chemicals: Recombinant Human maspin (rh Maspin) was a kind gift of Dr. Sally Twining, Dept. of Biochemistry, Medical College of Wisconsin, USA. ELISA maxisorp plates were obtained from NUNC, Denmark. Estrogen and other chemicals used were from Sigma Chemical Co.USA. ER (α and β) antibody was obtained from Thermo Fisher Scientific, NY, USA.

Preparation of Estrogen and Insulin solution: Estrogen and Insulin solution were prepared by dissolving the compounds in 0.9% NaCl, at pH 7.4.

Selection of patients with breast cancer and normal volunteers: Only female breast cancer patients between 35 and 65 years (mean 45 years, $n = 20$), participated in the study. None of them had received any therapy. Equal number of age matched normal female volunteers compared to that of the selected breast cancer subjects were asked to kindly participate in the study.

Determination of estrogen and progesterone receptor status of neutrophils from breast cancer subjects: ER statuses were determined by immunohistochemical techniques using fluorescence tagged antibodies and cells were observed and photographed. The neutrophils of the breast cancer subjects were classified as ER+ neutrophils or ER− neutrophils.

Collection of blood: The blood samples (20–25 ml) were collected by venipuncture and anticoagulated by gently mixing 1vol. of 0.13 M sodium citrate with 9 vol. of blood (Girish et al. 2006).

Immunization of the animals: Polyclonal antibodies against r-human maspin, estrogen, were raised by repeated immunization in White New Zealand rabbit (Tlaskalova-Hogenova and Stepankova 1980).

Assay of NO: Nitric oxide formation was assayed by methemoglobin method by following the protocol described before using Beckman Spectrophotometer (Model DU6) (Girish et al. 2006). The validity of the assay was confirmed by independent chemiluminescence method (Cox and Frank 1982).

Preparation of neutrophil suspension and the incubation of the isolated neutrophils with estrogen and insulin: Neutrophils isolated from the citrated blood samples suspended in HBBS buffer at pH 7.4 (6×109 cells/L) were incubated for 2.5 h at 23 °C with 200 μU of porcine insulin to reach equilibrium then were again incubated with different concentrations of estrogen and another set of neutrophils incubated with different concentrations of only estrogen for 4 h at 37 °C under sterile conditions (Klock and Bainton 1976).

In vitro *translation of maspin–mRNA*: In vitro translation of maspin–mRNA: Nucleic acids containing maspin mRNAs were isolated by Trizol methods from the neutrophils isolated from blood samples from breast cancer patients and from the normal volunteers (Cook et al. 2000). The nucleic acid preparation was incubated with ribosomal preparation, mixture of all amino acids (0.1 μmol each/ml) and 2 mM ATP as described (Zimmerman et al. 1979). After 6 h of incubation under sterile condition, the reaction mixture at 0 °C was centrifuged at 10,000 g for 10 min. The supernatant was used for the determination of maspin by ELISA as described below.

Enzyme linked immunosorbant assay (ELISA) for Maspin: Maspin was quantitated by ELISA using polyclonal antibody developed against rh Maspin (Girish et al. 2006). ELISA was performed by the method as described before (Engvall et al. 1972).

Scatchard plot analysis of the equilibrium binding of estrogen to its receptor in neutrophils: The neutrophils with the bound estrogen were separated from the unbound hormones by filtration over GF/C filter (Kahn and Sinha 1990b). The concentrations of estrogen in the sample were determined by ELISA. The dissociation constant (Kd) and the receptor numbers (n) were determined from Scatchard plots (Scatchard 1949).

Statistical analyses: The results obtained are presented as mean ± SD. The significance of the results was determined by Students' t-test, and $p < 0.005$ was considered to be significant.

3 Results

Determination of estrogen receptor subtype (α and β) in normal, ER+, ER– in peripheral neutrophils in blood: Immunohistochemical studies of the statuses of estrogen receptor in ER+ neutrophils and in the normal peripheral neutrophils demonstrated the presence of both α and β subtype of estrogen receptors. In contrast, the ER– neutrophils from the breast cancer patients showed the absence of both α and β subtype ER, suggesting pathophysiological defects in the synthesis of estrogen receptors proteins (α and β) in the peripheral neutrophils (Fig. 1).

Scatchard plot of the equilibrium binding of estrogen to intact normal neutrophils: Scatchard plot of the equilibrium binding of estrogen to normal neutrophils demonstrated typical homogeneous estrogen receptor population (Line A in Fig. 2). The analysis of the binding characteristics showed there were 4.179 ± 1.02 × 107 estrogen receptor binding sites/cell, with dissociation constant (Kd) 0.926 nM.

Detail of the equilibrium binding of estrogen to neutrophils was carried out as described in Materials and Methods. The estrogen was quantitated by ELISA using polyclonal antibody raised in rabbits as described in the Materials and Methods.

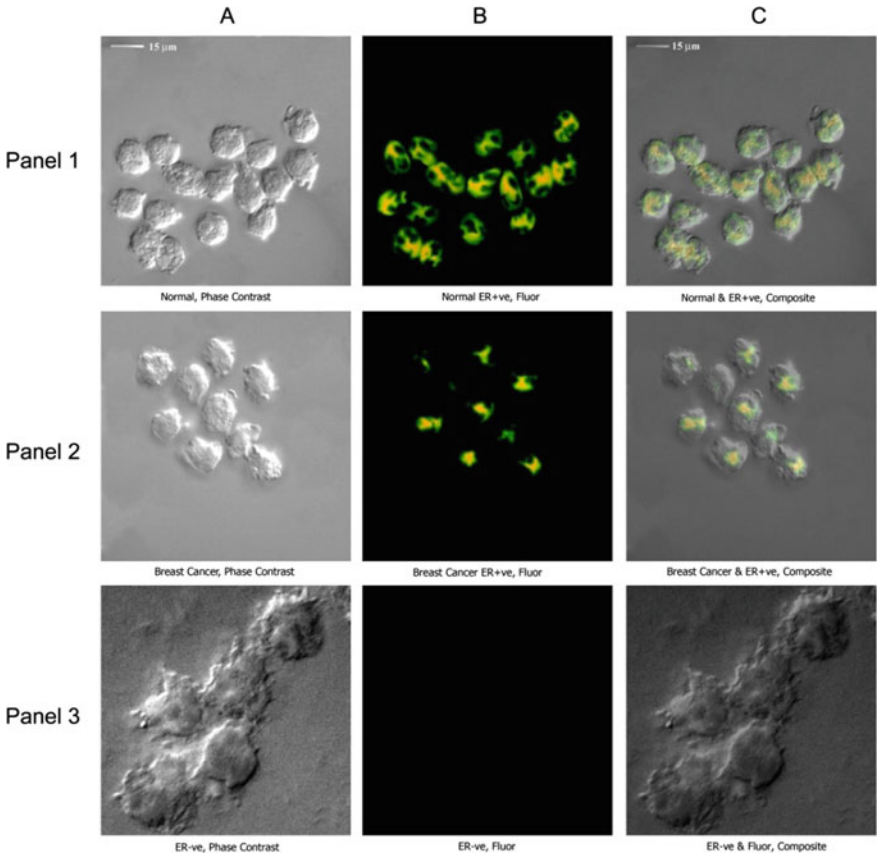

Fig. 1 Immunohistochemistry of estrogen receptors in neutrophils from normal and breast cancer patients: The panel 1 (A, B, C), panel 2 (A, B, C), panel 3 (A, B, C) represent normal (ER+) neutrophils, Breast Cancer (ER+) neutrophils and Breast Cancer (ER–) neutrophils, respectively. The figure presented is the typical representative of 6 more experiments using neutrophils from 6 different subjects from each group. The immunohistochemistry for estrogen receptor was determined as described in the Materials and Methods

In the experiment, the neutrophils were treated with 200 μunits of insulin for 2.5 h at 23 °C before these cells were used for the study of binding of estrogen without removing insulin from the cell suspension.

Effect of pre-incubation of neutrophils with insulin on the binding of estrogen to its receptors on these cells: Scatchard plot of the equilibrium binding of estrogen to the neutrophils pre-incubated with insulin was constructed (Line B in Fig. 2) and compared with that constructed using neutrophils that were not pre-incubated with insulin (Line A in Fig. 2). It was found that as a result of incubation of neutrophils with insulin the binding affinity for estrogen to its receptors in neutrophils remained essentially unchanged which demonstrated Kd = 1.072 nM compared to Kd of the

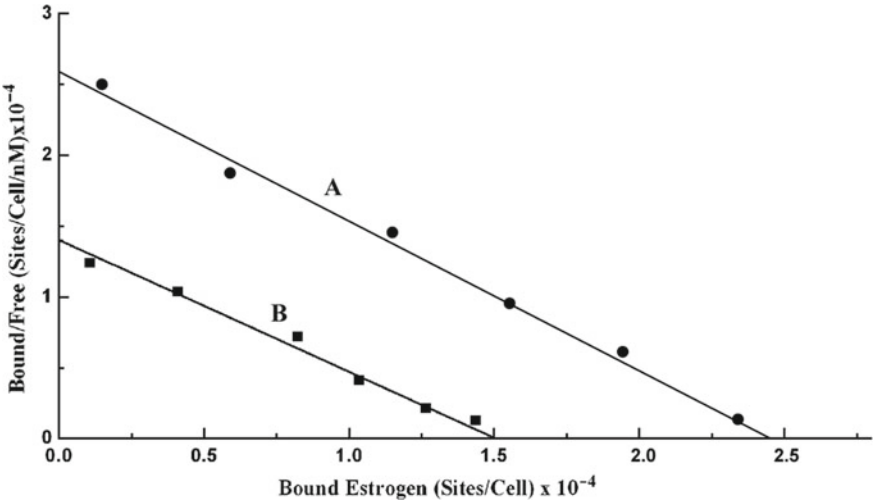

Fig. 2 Scatchard plot of estrogen binding to normal neutrophils pre-incubated with or without insulin: **a** Scatchard plot of equilibrium estrogen binding to normal neutrophils. **b** Scatchard plot of equilibrium estrogen binding to the normal neutrophils pre-incubated with insulin

binding of estrogen is 0.926 nM in the neutrophils that were not pretreated with insulin. The estrogen receptors which were 4.179 × 107/cell in the untreated cells was found to be decreased to 2.586 × 107/cell ($p < 0.005$, $n = 6$) after the same cell were treated with 200 μU of insulin.

The effect of estrogen on the synthesis of NO and maspin in normal ER+ and ER– neutrophils pre-incubated with insulin: It was found that the pre-incubation of normal ER+ neutrophils with insulin resulted in the significant impairment of the synthesis of both NO and maspin in these cells when compared to the control (Table 1). The treatment of normal ER– neutrophils which failed to produce either NO or maspin when treated with estrogen also failed to produce these agents when these cells were pretreated with insulin.

Table 1 The effect of estrogen on the synthesis of NO and maspin in normal ER+ and normal ER– neutrophils pre-incubated with insulin

Addition	Maspin (nM/6 × 10^9 cells)		NO (μM/ 6 × 10^9 cells)	
	ER+	ER–	ER+	ER–
Estrogen (0.6 nM) cells not pre-incubated with insulin	2.383 ± 0.014*	0	1.829 ± 0.072***	0
Estrogen (0.6 nM) + Insulin (200 μU) cells pre-incubated with insulin	1.454 ± 0.004*	0	0.889 ± 0.003***	0

($P < 0.005$, $n = 10$), ***($P < 0.005$, $n = 10$)

Table 2 The effect of estrogen on the synthesis of NO and maspin in breast cancer ER+ and Breast Cancer ER– neutrophils pre-incubated with insulin

Addition	Maspin (nM/6 × 10^9 cells)		NO (μM/ 6 × 10^9 cells)	
	ER+	ER–	ER+	ER–
Estrogen (0.6 nM) cells not pre-incubated with insulin	1.422 ± 0.029**	0	0.887 ± 0.003****	0
Estrogen (0.6 nM) + Insulin (200 μU) cells pre-incubated with insulin	0.790 ± 0.004**	0	0.470 ± 0.003****	0

($P < 0.005$, $n = 10$) ****($P < 0.001$, $n = 10$)

The effect of estrogen on the synthesis of NO and maspin in Breast Cancer ER+ and ER– neutrophils pre-incubated with insulin: It was found that the pre-incubation of breast cancer ER+ neutrophils with insulin resulted in the significant furthermore impairment of the synthesis of both NO and maspin in these cells when compared to the control (Table 2). The treatment of breast cancer ER– neutrophils which failed to produce either NO or maspin when treated with estrogen also failed to produce these agents when these cells were pretreated with insulin.

4 Discussions

Hormones bind to their specific receptors on the cell surface to accomplish their specific physiological effects in the target cells. Ligand (hormone)-receptor binding results in the expression of the hormone effects. The negative cooperativity or positive cooperativity between the hormone receptors may significantly either down-regulate or up-regulate respectively the activity of unrelated hormones in the system (De Meyts et al. 1978). Insulin, a hypoglycemic hormone has its specific functions, in this case influenced the activity of estrogen which is known to induce the synthesis of maspin, an anti-breast cancer protein through the NO synthesis in normal as well as from the breast cancer neutrophils (Ganguly Bhattacharjee et al. 2012). ER- reported to produce worse prognostic outcome of the disease when compared to that in ER+ breast cancer neutrophils (Kiba et al. 2008).

It has been reported that ER+ neutrophils from the breast cancer patients produced less amount of NO induced maspin synthesis compared to normal control (Ganguly Bhattacharjee et al. 2012). The pre-incubation of ER+ neutrophils with insulin resulted in the attenuation of estrogen receptor number in the intact neutrophils and declined NO induced maspin production by estrogen.

Our results implied that the systemic presence of insulin might adversely affect the systemic production of the anti-breast cancer protein due to the insulin induced heterologous downregulation of the steroid receptor numbers in the neutrophils that

not only resulted in the declined estrogen induced maspin synthesis, but also inhibited NO synthesis induced by the steroid in these cells.

It may be speculated that in Type 2 diabetes mellitus which causes hyperinsulinemia due to systemic insulin resistance might actually lead to worse prognostic outcome in breast cancer. On the other hand in Type 1 diabetes where insulin synthesis is completely impaired might be beneficial in breast cancer in terms of maspin synthesis (Hojo et al. 2001; Liu et al. 1999b; Berg et al. 1987; Jazieh et al. 2004).

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