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ORIGINAL ARTICLE

Investigations on the inducible and endothelial nitric oxide synthases in human breast cancer cell line MCF-7 – estrogen has an influence on e-NOS, but not on i-NOS

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Abstract

As a model for hormone-dependent breast cancer, we studied the MCF-7 cell line to examine differences in the stimulation of the inducible (i) and endothelial (e) nitric oxide synthase (NOS) and the role of 17β-estradiol (E_2).

MCF-7 cells were stimulated with (a) E_2 (10^{-8} M) and (b) a combination of different cytokines such as interleukin-1 beta (Il-1β), tumor necrosis factor alpha (TNF-α) and interferon gamma (INF-γ), and lipopolysaccharide (LPS). e-NOS and i-NOS proteins were measured using Western blot analysis. Using the Griess method nitric oxide (NO) was estimated by assessing the stable product nitrite (NO_{2-}) in the culture medium, and a direct method, employing EPR spin trapping also was used. Western blot analysis revealed the presence of e-NOS and i-NOS in MCF-7 cells. In Western blot analysis, e-NOS, but not i-NOS, expression could be stimulated by E_2. An increase in NO_{2-} was noted after stimulation of MCF-7 using different combinations of cytokines Il-1β, TNFα and INFγ, and LPS, but not after E_2.

In conclusion, e-NOS and i-NOS are weakly expressed in the MCF-7 cell line, but are stimulated differently. The MCF-7 cell may contain both a constitutive NOS and an inducible NOS.

Keywords: Breast cancer; Nitric oxide synthases; Nitric oxide; MCF-7; Estradiol

Introduction

Nitric oxide (NO) is an inorganic free radical acting as an intracellular and extracellular messenger released in mammalian cells. It has many different functions depending on the tissues and plays an important role in tumor biology that still is not fully identified [22]. It is produced by nitric oxide synthase (NOS), which catalyzes the conversion of L-arginine to L-citrulline and NO. Three isoforms of NOS have been identified so far. The neuronal (n) and the endothelial (e) NOS are constitutively expressed and are calcium-dependent. The inducible (i) NOS is calcium-independent. The i-NOS

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was found to be expressed in response to cytokines and lipopolysaccharide (LPS). Many cancer cell lines have been shown to express NOS [17,38].

In our study, investigating human breast cancer and in situ lesions of the breast, but not benign lesions, NOS (e-NOS and i-NOS) and NO could be detected [20]. This expression seems to be related to tumor differentiation and steroid receptor status [19,21]. The results still are conflicting, and the relationship between NOS and breast cancer has not yet been established.

Hormones and cytokines are able to interfere with the NO production [24,34,37].

There is now accumulating evidence that E_2 treatment can increase NOS expression in a wide range of tissues [8,36]. Because breast cancer is a hormone-dependent disease, there might be an influence on NOS expression.

The purpose of this study was to track the e-NOS and i-NOS expressions in the hormone-dependent breast cancer cell line MCF-7 and to examine the role of the estrogen 17β-E_2. While an effect of E_2 on i-NOS expression was not noted in our study, cytokines such as interleukin 1-beta (Il-1β) and tumor necrosis factor alpha (TNF-α), interferon gamma (INF-γ), and LPS can affect the production of NO.

Materials and Methods

Drugs

IL-1β was obtained from Collaborative Research Inc (Bedford, MA, USA); INF-γ, LPS, E_2, and a monoclonal antibody directed against β-actin were purchased from Sigma (Deisenhofen, Germany); TNF-α was obtained from Boehringer (Ingelheim, Germany). Dulbecco's modified Eagle's medium (DMEM), glutamine, penicillin/streptomycin, and trypsin were purchased from Invitrogen (Karlsruhe, Germany) and fetal calf serum (FCS) from PAA Laboratories (Linz, Austria). The polyclonal antibody directed against i-NOS was kindly provided by Prof. J. Pfeilschifter (Department of Pharmacology, University of Frankfurt). The monoclonal antibody directed against e-NOS was purchased from Transduction Laboratories (Heidelberg, Germany). Secondary antibodies were obtained from Santa Cruz (Heidelberg, Germany).

Cell culture

The breast cancer cell line MCF-7 was obtained from DMSZ (Braunschweig, Germany) and routinely cultured in DMEM with phenol red, 10% FCS, antibiotics, and 2 mM L-glutamine in an atmosphere of 5% CO_2 in humidified air at 37 °C. Human umbilical vein endothelial cells (HUVECs, passage 4) were kindly provided by Ms. Oppermann (Department of Surgery, University of Frankfurt) and routinely cultured in RPMI-1640 containing 15% FCS, 2 mM L-glutamine, 1% penicillin/streptomycine (P/S), and endothelial cell growth supplement (ECGS) (Calbiochem, Bad Soden, Germany) in an atmosphere of 5% CO_2 in humidified air at 37 °C. Smooth muscle cells from rat aorta were obtained by the explant technique using small pieces of aortic media obtained from Wistar rats. Smooth muscle cells were cultured in MEM containing 10% FCS, 2 mM L-glutamine, and antibiotics. When cells reached confluence, they were serum-deprived using MEM containing 0.1% fatty-acid-free bovine serum albumin, 2 mM L-glutamine, and antibiotics for 24 h.

Western blot analysis

Following treatment with E_2 for 24 and 48 h, MCF-7 cells were lysed for subsequent Western blotting as described previously [6,31]. Membranes were incubated with either an antibody directed against β-actin (dilution 1: 200,000), e-NOS (dilution 1:2000), or i-NOS (dilution 1:1000) as indicated for 1 h. After washing, the membranes were incubated with a secondary goat anti-mouse antibody conjugated to horseradish peroxidase (dilution 1:2000) for 30 min to detect endothelial NOS and β-actin immunoreactive bands, and with a goat anti-rabbit antibody (dilution 1:2000) to detect inducible NOS immunoreactive band. Endothelial NOS, i-NOS, and β-actin immunoreactivity was visualized by exposing Western blot membranes to an X-ray film to blots incubated with the ECL reagent (Pierce, Rockford, USA). Quantitative analysis of immunoreactive bands was performed using a Kodak gel documentation system (1D 3.5). The expression level of e- and i-NOS was compared with that of the standardized β-actin expression level.

Release of nitrite

The formation of NO was indirectly assessed by measuring the accumulation of NO_{2-} in the cell culture medium over a 24-h treatment period. MCF-7 cells were plated into 24-well plates and allowed to grow for 24 h. Thereafter, the medium was replaced with fresh culture medium without FCS in the absence or presence of either E_2 (10^{-8} M) or a mixture of cytokines (TNF-α: 1 U/ml; IFN-γ: 500 U/ml; IL-1β: 100 U/ml, and LPS: 10 µg/ml). The nitrite concentration in the conditioned medium was determined using the Griess reaction with a standard curve based on the measurement of $NaNO_2$ [14].