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Original Research

Antagonism of LPS and IFN-γ induced iNOS expression in human atrial endothelia by morphine, anandamide, and estrogen

Caterina FIMIANI[1], Harold MAGAZINE[2], Ingeborg D WELTERS[1], Thomas V BILFINGER[1,3], Felice SALSANO[4], Else TONNESEN[5], George B STEFANO[1,3,6] ([1]*Neuroscience Research Institute, State University of New York, College at Old Westbury, Old Westbury, NY 11568 USA*；[2]*Queens College and Graduate School of the City University of New York, Flushing, New York 11367, USA*；[3]*Division of Cardiothoracic Surgery, Department of Surgery, State University of New York, College at Stony Brook, Stony Brook, NY 11794, USA*；[4]*Clinical Medicine Department, La Sapienza University, Rome, Italy*；[5]*Department of Anesthesia, Aarhus Kommunehospital, Denmark*)

KEY WORDS nitric oxide；morphine；anandamide；estrogens；nitric oxide synthase；human atria；endothelium

ABSTRACT

AIM：To determine whether inducible nitric oxide synthase (iNOS) stimulation of human atrial fragments can be diminished by the naturally occurring signal molecules, such as morphine, anandamide, and estrogen. The use of iNOS as an indicator is justified since it has been associated with initiation of various types of cellular damage either directly or indirectly. **METHODS**：Western blots were performed on control and drug-exposed atrial tissue before and after lipopolysaccharide (LPS) and interferon-γ (IFN-γ) exposure. **RESULTS**：Preincubation of the tissue with morphine, anandamide or estrogen prior to, but not after, the addition of LPS + IFN-γ, blocked iNOS expression. The nitric oxide donor SNAP also blocked iNOS induction while preincubation of atrial fragments with an inhibitor of NOS, *L*-NAME, prior to morphine or anandamide exposure, restored LPS + IFN-γ induction of iNOS. **CONCLUSION**：These data suggest a direct regulatory link at the transcriptional level between constitutive (c) NOS and iNOS in human atrial tissue.

INTRODUCTION

In light of our earlier studies of constitutive nitric oxide synthase (cNOS)[1-9] stimulation by the naturally occurring nitric oxide (NO) stimulators, morphine, anandamide and estrogen, it became important to examine the regulatory relationship between cNOS and inducible iNOS activation or inhibition in human atrial endothelial tissue. This is important since the downregulation of iNOS expression may diminish many deletrious processes associated with its prolonged activity[10,11].

The present study demonstrates for the first time that morphine, anandamide, and 17β-estradiol inhibit lipopolysaccharide (LPS)-and interferon-γ (IFN-γ)-induced iNOS expression in atrial endothelial cells. Thus, iNOS expression can be down regulated as a result of morphine, anandamide, or estrogen induced cNOS activation, demonstrating a regulatory link between cNOS and iNOS activation. This finding is significant because it strongly suggests the use of morphine as an anesthetic agent since it can downregulate iNOS expression and it partially may explain the biologically beneficial biomedical effects of estrogen. Thus, the present study sought to determine if these naturally occurring signal molecules can downregulate iNOS expression, demonstrating their significance in clinical situations requiring this action, ie, surgery.

MATERIAL AND METHODS

Atrial preparation Atrial fragments (6-mm pieces) were obtained from patients undergoing elective coronary artery bypass grafting (CABG) for atherosclerotic coronary artery disease as these tissues are regarded as waste during atrial cannulation. The project was approved by the institutional review board. Patients with

[6] Correspondence to Dr George B STEFANO. Phn 516-876-2732. Fax 516-876-2727. E-mail stefanog@surg.som.sunysb.edu
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chronic illnesses, such as diabetes or cancer as well as acute processes (eg, known infections, trauma) were excluded. In all patients undergoing CABG, the anesthetic management included induction with fentanyl (fentanyl citrate adjusted for pH) up to 15 μg/kg. Maintenance was achieved with the same agent. It is important to note that fentanyl does not influence the μ_3 opiate receptor[12] and, thus, does not share immunosuppressive and NO-stimulating actions with morphine[2,3,12,13]. The atrial fragments were stored in an electrolyte solution at 4 ℃ (500 mL plasmalyte with 5000 U heparin and 60 mg papaverine), and these specimens were immediately transported on ice to the laboratory for processing. Immediately upon arrival, each fragment was washed in phosphate buffered saline (PBS) and cut into 2-mm square pieces and then placed into a superfusion chamber[14] endothelial side up, filled with 2 mL of PBS.

Superfusion Atrial fragments were incubated in a plexiglass perfusion chamber[7,14] containing 1 mL of RPMI maintained at 5 % CO_2, 37 ℃ for 40 min. After the initial incubation, the fragments were superfused with PBS by a four-channel peristaltic pump (Rainin, Woburn, MA) at a flow rate of 0.2 ml/min to an inflow opening at the bottom of the chamber. The outflow tube was located opposite the inflow tube at the top of the cylindrical chamber. This arrangement allowed total superfusion of the tissue fragments. The perfusing solution was altered at the indicated intervals by manually transferring the inflow tubing to the appropriate beaker containing the various agents, ie, morphine *etc*, at predetermined concentrations and the superfusate was removed from the chamber through outflow tubing connected to the outflow tube near the rim of the chamber. For drug exposure the inflow tubing was connected to a respective vial containing the study drug and then returned to the vehicle, buffer minus the drug, for the remainder of the experiment.

Western blot analysis Control and drug-exposed atrial fragments, as noted above, were separately washed twice with ice-cold PBS. They were homogenized by Polytron (Brinckman instruments) in 5 volumes of ice-cold lysis/suspension buffer [NaCl 100 mmol/L, Tris-HCl 10 mmol/L pH 7.6, edetic acid 1 mmol/L pH 8, aprotinin 1 mg/L, phenylmethylsulfonyl fluoride (PMSF) 100 mg/L]. Tissue lysates were transferred to microcentrifuge tubes, incubated on ice for 30 min and centrifuged at 14 000 × g for 30 min at 4 ℃. The supernatant fluid (total cell lysate) was used for Western blot analysis. Western blot analysis was carried out using 50 μg of the total tissue lysates. Protein were electroblotted from SDS-polyacrilamide gel onto nitrocellulose membranes. The membrane was blocked with a solution of bovine serum albumin, goat IgG and Tween 20 (membrane blocking solution from ZYMED Laboratories, Inc, San Francisco, CA) for 2 h at room temperature. Rabbit polyclonal antisera (1∶1 000 dilution in the above blocking solution) to iNOS were from Santa Cruz Biotechnology (Santa Cruz, CA). The blot was incubated with the primary antibody for 2 h at room temperature and then washed three times with PBS-0.05 % Tween 20. The filter was developed with an alkaline-phosphatase conjugated secondary antibody (1∶100 dilution) and a BCIP/NBT chromogen/substrate solution according to manufacturer's instructions (Immunoblot-AP-Kit, ZYMED Laboratories). Atrial fragments where the endothelium was scraped off did not exhibit the respective-down regulation of iNOS expression. The images were captured as digital images via SONY 3 chip digital camera and analyzed via Gel Pro Density Analysis (Media Cybemetrics, Inc, MD).

All agents were purchased from Sigma (St Louis, MO).

RESULTS

As noted in previous reports, exposing saphenous vein, internal thoracic artery or atrial endothelial cells to either morphine, anandamide or estrogen results in cNOS-coupled NO release that is concentration dependent and antagonisable by the respective antagonists[2,3,5,8,15-18].

Using the previously determined efficacious doses of LPS (1 mg/L) and IFN-γ (150 kU/L)[7], we demonstrated that both agents induced the expression of iNOS (Fig 1). We next analyzed the effects of morphine, anandamide (both agents at 1 μmol/L for 10 min) and 17β-estradiol on iNOS expression. In atrial fragments pre-exposed to morphine, anandamide or 17β-estradiol (1 nmol/L); subsequent addition of LPS (1 μg) and IFN-γ (150 kU/L) to the medium, failed to stimulate iNOS expression (Fig 1). This inhibition of iNOS expression was blocked by first exposing the tissue to the NOS inhibitor L-NAME (0.1 mmol/L; Fig 1). In this regard, given the fact that anandamide, morphine and estrogen receptors are coupled to cNOS-NO release, we exposed these tissues to the NO-donor, *S*-nitroso-*N*-acetyl-*DL*-penicillamine (SNAP; Sigma, St Louis, MO) at a level of 30 nmol/L, since this concentration represents the lev-

el of NO release stimulated by morphine in this tissue, and then LPS + IFN-γ. SNAP, in this experiment, inhibited the expression of iNOS, supporting the hypothesis that cNOS stimulated NO suppresses iNOS expression[7].

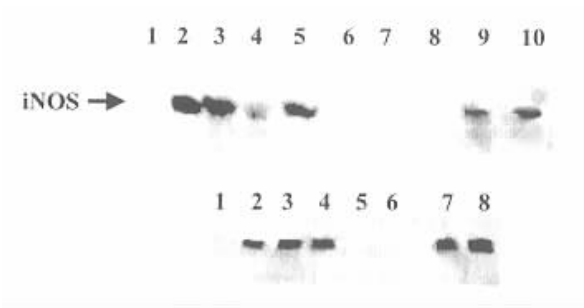

Fig 1. Western blot analysis of iNOS protein expression after LPS/INFγ and anandamide/morphine/17β-estradiol incubation. Top: Lane 1: unstimulated atrial tissue. Lane 2: Atrial tissue stimulated with 1 mg/L LPS for 1 h. Lane 3: Atrial tissue stimulated with 1 mg/L INFγ for 1 h. Lane 4: Pretreatment with LPS (1 mg/L) followed by incubation with anandamide (1 μmol/L) for 10 min. Lane 5: Pretretment with LPS (1 mg/L) followed by incubation with morphine (1 μmol/L) for 10 min. Lane 6: Pretreatment with morphine (1 μmol/L) for 10 min followed by incubation with LPS (1 mg/L). Lane 7: Pretreatment with anandamide (1 μmol/L) for 10 min followed by incubation with LPS (1 mg/L). Lane 8: Treatment with SNAP (30 nmol/L) followed by LPS (1 mg/L) and *L*-NAME (0.1 mmol/L) 2 min prior to morphine addition. Lane 9: Treatment with *L*-NAME (0.1 mmol/L) then morphine followed by LPS exposure. Lane 10: Control protein. Bottom: Lane 1: unstimulated atrial tissue. Lane 2: Atrial tissue stimulated with 1 mg/L LPS for 1 h. Lane 3: Atrial tissue stimulated with 1 mg/L LPS + 150 kU/L INFγ for 1 h. Lane 4: Preatreatment with LPS (1 mg/L) followed by incubation with 17-β estradiol (1 nmol/L) for 10 min. Lane 5: Pretreatment with 17-β estradiol (1 nmol/L) for 10 min followed by incubation with LPS (1 mg/L). Lane 6: Treatment with SNAP (30 nmol/L) followed by LPS (1 mg/L) and L-NAME (0.1 mmol/L) 2 min prior to 17-β estradiol addition. Lane 7: Treatment with *L*-NAME (0.1 mmol/L) then 17-β estradiol followed by LPS exposure. Lane 8: Control protein

DISCUSSION

The present study demonstrates the following: 1) LPS and IFN-γ stimulate iNOS expression in human atrial endothelia; 2) Prior exposure to morphine, anandamide and 17β-estradiol block the LPS- and IFN-γ-induced iNOS expression, whereas treatment in the reverse order does not; 3) SNAP also inhibits iNOS expression; and lastly 4) cNOS NO is linked to iNOS expression, suggesting that morphine, anandamide and estrogen may be used to down regulate iNOS expression under clinical circumstances. Taken together, the study strongly suggests that these naturally occurring ligands can have beneficial biomedical actions based on their ability to stimulate cNOS activity and inhibit iNOS.

The biomedical significance of this finding may be found in surgical trauma that induces an excitatory immune response, ie, diffuse or local inflammatory response, triggered by the proinflammatory cytokines namely, tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and IL-8[19]. This proinflammatory primary immune response is followed by a second down regulating activation by anti-inflammatory mediators such as IL-10 and morphine[19-23]. Based on the common inhibitory activities of IL-10 and morphine on immunocytes and endothelial cells, a common mechanism involving NO coupling and release was proposed[23]. Thus, in conditions with excessive release of proinflammatory mediators such as vascular trauma or systemic inflammatory response syndrome, such as evoked by cardiopulmonary bypass[2], endogenous morphine or anandamide may act synergistically with IL-10 to restore normal levels of neural and immune activity/homeostasis. The present report extends this hypothesis to include estrogen. Supporting this hypothesis that these specific intercellular signalling molecules may be of singular importance via a constitutive NO-mediated mechanism is a recent study demonstrating that IL-10 suppressed NF-κB activation through preservation of IκBα *in vivo*[24].

In summary, excessive induction of iNOS is known to contribute to systemic hypotension, myocardial depression, and vasodilation as seen in critical illness[10]. In a rat model of septic shock, inhibition of NF-κB activation by pyrrolidine dithiocarbamate prevented systemic hypotension and reduced LPS-induced iNOS expression[11]. Once cNOS is stimulated, iNOS synthesis reportedly cannot be induced within a specific period of time[13,25,26]. This coupled with our present data demonstrates that within a certain time frame there is only one of two possible outcomes, either activation of cNOS or iNOS. The data also suggest, as first observed by Peng and colleagues[27], that cNOS may exert a tonic inhibition of iNOS, resulting in limiting NO levels. Furthermore, as strongly suggested by these studies, compounds such as morphine, ananadamide, IL-10 and estrogen, we surmise, may exert common immunosuppressory actions via

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NO effect on NF-κB activation[28]. The fact that these same signalling molecules exert the same actions in invertebrate cells indicates a basic very old biological strategy to limit immune activation. Simultaneously, these studies call for a re-evaluation of substances of abuse, such as morphine and cannabinoids, as they represent a naturally occurring mechanism to down regulate immunoresponsiveness in desired conditions. A thorough investigation of their role at the local endovascular level in the initiation of healing of endothelial injury is warranted. Clearly, we demonstrate that these naturally occurring signal molecules down regulate iNOS expression and thus their use, especially of morphine as an anesthetic, in specific clinical situations, such as major surgery, may be warranted.

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脂多糖和干扰素-γ 拮抗吗啡，anandamide，和雌激素诱导的人心房内皮 iNOS 的表达

Caterina FIMIANI[1]，Harold MAGAZINE[2]，Ingeborg D WELTERS[1]，Thomas V BILFINGER[1,3]，Felice SALSANO[4]，Else TONNESEN[5]，George B STEFANO[1,3,6]

关键词 一氧化氮；吗啡；anandamide；雌激素类；一氧化氮合酶；人类心房；内皮

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