Ph.D. THESIS

ÉVA MORSCHL

INTERACTIONS OF ESTROGEN AND NITRIC OXIDE IN THE CIRCULATORY HOMEOSTATIC CONTROL

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CONTENTS

Own articles, the thesis based on[1]:

I. **Morschl É.**, Bretus I., Pávó I., Topa L., Weiszhár Zs., László F. Nitric oxide-mediated mucus hypersecretion protects the stomach of ovariectomized rats. Eur. J. Pharmacol. 2000;392:R5-R7.

II. **Morschl É.**, Bretus I., Nemcsik J., László F., Pávó I. Estrogen-mediated up-regulation of the Ca-dependent constitutive nitric oxide synthase in the rat aorta and heart. Life Sci. 2000;68(1):49-55.

III. Pávó I., László F., **Morschl É.**, Nemcsik J., Berkó A., Cox D.A., László F.A. Raloxifene, an estrogen-receptor modulator, prevents decreased constitutive nitric oxide and vasoconstriction in ovariectomized rats. Eur. J. Pharmacol. 2000; 410(1):101-104.

IV. **Morschl É.**, Pávó I., Nemcsik J., Varga G., László F., Whittle B.J.R. Endogenous bacteria-triggered inducible nitric oxide synthase activation protects the ovariectomized rat stomach. J. Physiol. (Paris) 2001;95(1-6):137-140.

Own articles, the thesis related to[2]:

1. László F., **Morschl É.**, Pávó I., Whittle B.J.R. Nitric oxide modulates the gastrointestinal plasma extravasation following intraabdominal surgical manipulation in rats. Eur. J. Pharmacol. 1999;375(1-3):211-215.

2. Szepes Z., **Morschl É.**, Kiss J., Pávó I., Whittle B.J.R., Varga Cs., László F.A., László F. Detrimental effects of oestradiol on cysteamine-induced gastroduodenal ulceration in the female rat. J. Physiol. (Paris) 1999;93:491-494.

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[1]Articles of which results are fully demonstrated and discussed in the thesis
[2]Articles of which results are related to the thesis and cited in the text where appropriate

3. Pávó I., **Morschl É.**, Szepes Z., Kiss J., Boda K., Vetró G., Varga Cs., László F.A., László F. Vasopressin deficiency decreases the frequency of gastroduodenal ulceration in humans J. Physiol. (Paris) 2000;94:63-66.

4. Pávó I., Pozsár J., **Morschl É.**, Nemcsik J., László F., Whittle B.J.R. Interactions of pro-inflammatory and vasoactive mediators with nitric oxide in the regulation of rat vascular permeability during laparotomy. Eur. J. Pharmacol. 2000;402(1-2):193-197.

5. Kiss J., Lamarque D., Moran A.P., Pozsár J., **Morschl É.**, László F., Whittle B.J.R. *Helicobacter pylori* lipopolysaccharide-provoked injury to rat gastroduodenal microvasculature involves inducible nitric oxide synthase. Eur. J. Pharmacol. 2001;420(2-3):175-179.

6. László F.A., Varga Cs., Pávó I., Gardi J., Vecsernyés M., Gálfi M., **Morschl É.**, László F., Makara G.B. Vasopressin pressor receptor-mediated activation of the HPA axis by acute ethanol stress in rats. Am. J. Physiol. 2001;280:R458-R465.

7. Szepes Z., Kiss J., Lamarque D., Moran A.P., Nemcsik J., **Morschl É.**, László F., Whittle B.J.R. Attenuation of Helicobacter pylori endotoxin provoked rat intestinal inflammation by selective inhibition of the inducible nitric oxide synthase. J Physiol-Paris 2001;95(1-6):453-455.

8. Whittle B.J.R., **Morschl É.**, Pozsár J., Moran A.P., László F. *Helicobacter pylori* lipopolysaccharide provokes iNOS-mediated acute systemic microvascular inflammatory responses in rat cardiac, hepatic, renal and pulmonary tissues. J. Physiol. (Paris) 2001;95(1-6):257-259.

List of abbreviations

cNOS	constitutive nitric oxide synthase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GMP	guanidine monophosphate
iNOS	inducible nitric oxide synthase
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
L-NAME	N^G-nitro-L-arginine methyl ester
L-NNA	N^G-nitro-L-arginine
LPS	lipopolysaccharide
THB_4	tetrahydrobiopterin

1. INTRODUCTION

1.1. The vascular endothelium

Endothelium is a cell monolayer, which constitutes the internal structure of the entire circulatory system. It has long been considered as a "cling film", which at most prevents coagulation and serve as an anatomical barrier between blood and interstitium throughout the vascular tree. Endothelium also expresses cell adhesive molecules, which drive the adhesion and subsequent trans-endothelial migration of leukocytes into the intima. Generally, the endothelium controls the inter- and transcellular traffic of numerous nutrients, hormones, and cells. It also acts as a local regulator adapting blood flow to local metabolic needs. A variety of endothelium-derived relaxing and contracting factors such as nitric oxide (NO), prostacyclin, endothelium-derived hyperpolarizing factor, endothelin and thromboxan A2 play a role in the control of vascular tone (Fig.1.) [Lüsher and Barton, 1997; Arnal et al., 1999].

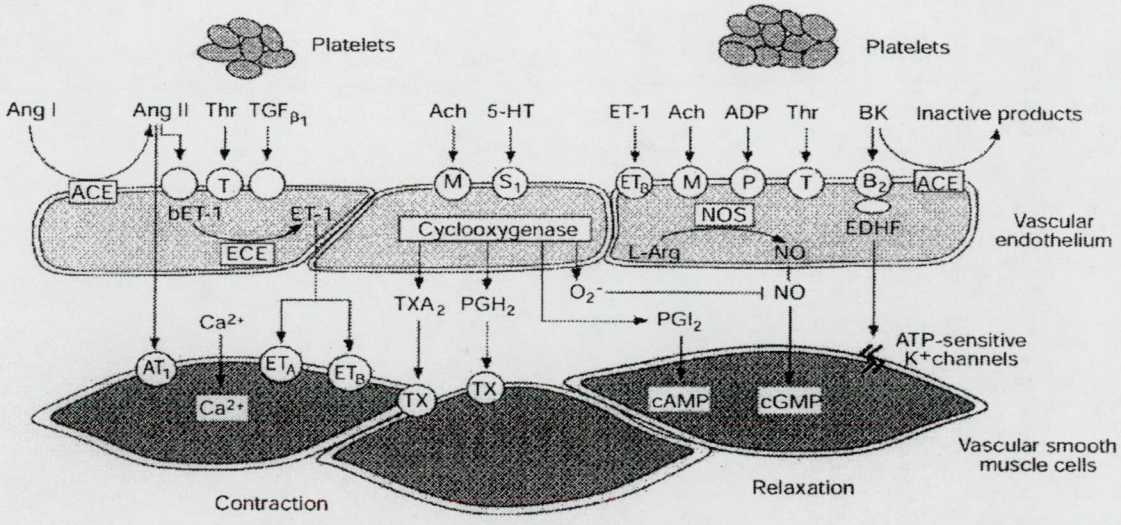

Fig. 1.

Vasoactive mediators released by the endothelium. Ang-angiotensin, ACE-angiotensin-converting enzyme, Ach-acetylcholine, ADP/ATP-adenosine diphosphate/triphosphate, Bk-bradykinin, cAMP/cGMP-cyclic adenosine/guanosine monophosphate, ECE-endothelin-converting enzyme, EDHF-endothelium derived hyperpolarizing factor, ET-endothelin 1, 5HT-5-hyroxytryptamine (serotonin), L-Arg-L-arginine, NO-nitric oxide,NOS-nitric oxide synthase, O^{2-}-superoxide, PGH_2-prostaglandin H_2, PGI_2-prostacyclin, $TGF\beta_1$-transforming growth factor β_1, Thr-thrombin, TXA_2-thromboxane A_2. Circles represent receptors: AT-angiotensinergic, B-bradykinergic, ET-endothelin receptor, M-muscarinic, P-purinergic, S-serotonergic, T-thrombin receptor, TX-thromboxane receptor. (by Lüscher T.F. and Barton M. Clin Cardiol 1997;20:Suppl.II.3.)

Many diseases have been reported to be associated with an impared endothelium-dependent vasodilatation which may contribute to an increased susceptibility to vasospasm, decreased inhibition of thrombus formation and an impaired ability to reduce vascular resistance in ischaemic conditions.

1.2. Nitric oxide synthase enzyme isoformes

Robert Furchgott and John Zawadski recognized the importance of the endothelium in acetylcholine-induced vasorelaxation in 1980 than later Luis Ignarro and Salvador Moncada identified endothelial-derived relaxing factor as NO [Furchgott and Zawadski, 1980; Ignarro et al., 1987; Palmer et al., 1987]. NO has a half-life of only a few seconds *in vivo.* However, since it's soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes. In the vasculature, NO reacts with iron in the active site of the enzyme guanylate cyclase, stimulating it to produce the intracellular mediator cyclic GMP, that in turn enhances the release of neurotransmitters resulting in smooth muscle relaxation and vasodilation. NO is synthetized from L-arginine (Fig.2.) by a family of enzymes termed NO synthase (NOS, EC 1.14.13.39).

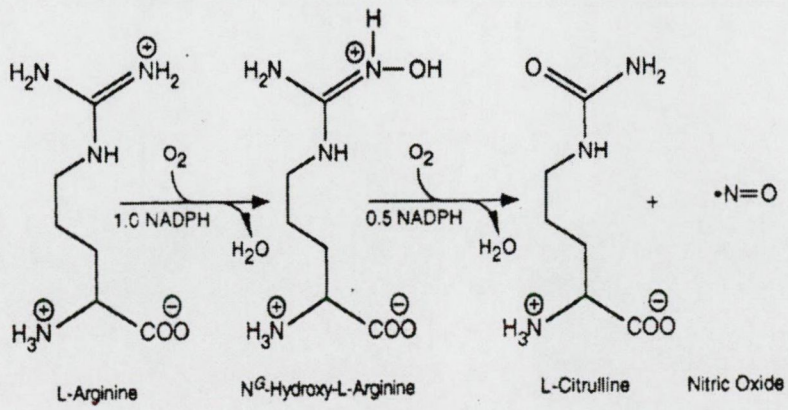

Fig. 2.

The NOS catalysed reaction. (by Andrew P.J., Mayer B. Cardiovascular Research 1999;43:522.)

There are three distinct isoformes of NOS. Endothelial NOS (eNOS or NOS III) and neuronal NOS (nNOS or NOS I) are generally referred to as constitutively expressed, Ca^{2+}-dependent enzymes (cNOS). Inducible NOS (iNOS or NOS II) is expressed mainly in pathological states

e.g. following cytokine or bacterial endotoxin exposure and its activity is independent of the increase of intracellular Ca^{2+}-level (Table 1.).

Table 1. The three isoforms of NOS. (by Arnal J.F. et al., Cell Mol Life Sci 1999;55:1079.)

Specificity	Endothelial (III)	Neuronal (I)	Inducible (II)
Molecular mass	135 kD	155 kD	130 kD
Function of NO	endothelium-derived relaxing factor; antiaggregant	neurotransmitter; neuromodulator; relaxation of smooth muscle	non-specific; host defense
Cofactor Ca^{2+}/calmodulin	dependent	dependent	independent
Other cofactors: NADPH, FAD, FMN, tetrahydrobiopterin	dependent	dependent	dependent
Stimuli	acetylcholine; bradykinin; serotonin; ATP; shear stress	neuro-excitatory amino acids	?
Mechanisms of regulation (+activation, − inhibition)	$+Ca^{2+}$/calmodulin protein interaction (Cav-1, +HSP 90) + dimerization ? phosphorylation	$+Ca^{2+}$/calmodulin protein interaction + dimerization ? phosphorylation	+ dimerization phosphorylation
Regulation of gene expression (+activation − inhibition)	+shear stress +proliferation −tumor necrosis factor-α (TNF-α) ? estrogens	+ estrogens	+interleukin-1, +interferon-γ, +TNF α −transforming growth factor β +AMPc, GMPc +NFκB −NO −glucocorticoids
Mechanisms	transcription; mRNA stability	transcription	transcription; mRNA stability

These enzymes function as a homodimer consisting of two identical monomers, which can be functionally and structurally divided into a C-terminal reductase and an N-terminal oxygenase domain. The reductase domain contains binding sites for NADPH, FAD and FMN, whereas the oxygenase domain binds haem, TBH_4 and the substrate L-arginine. Between the two domains lies the calmodulin (Ca^{2+}-binding protein) binding region, which plays a key role in both the structure and function of the enzyme (Fig.3.) [Andrew and Mayer, 1999].

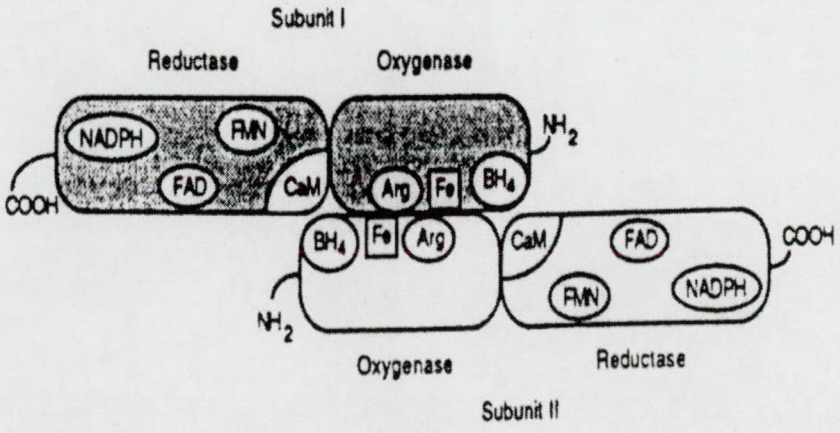

Fig. 3.

Scheme of the domain structure of the NOS dimer, showing cofactor and substrate binding sites. NADPH-nicotinamide adenine dinucleotide phosphate, FAD-flavin adenine dinucleotide,FMN-flavin mononucleotide, BH_4-tetrahydrobiopterin, CaM-calmodulin, Arg-arginine, Fe-iron (by Andrew P.J., Mayer B. Cardiovascular Research 1999;43:522.)

The main source of endothelial NO is eNOS expressed by endothelial cells, but the enzyme is also expressed in cardiomyocytes, blood platelets and cardiac conduction tissue. Although the enzyme does not contain any hydrophobic transmembrane domain, it is targeted to plasmalemmal caveolae, which are small invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin. Caveolin-1 has an inhibitory effect on eNOS activity and this can be completely reversed by Ca^{2+} /calmodulin, a complex elicited after the stimulation of endothelial cells by several agonists (e.g. catecholamines, vasopressin, bradykinin, histamine, serotonin and ADP). A possible mechanism is that the activated eNOS-calmodulin complex synthetizes NO until the intracellular free Ca^{2+} concentration decreases to the point where the calmodulin dissociates and the inhibitory eNOS-caveolin-1 complex reforms [Arnal et al., 1999]. The availability of L-arginine is one of the rate-limiting factors in cellular NO production. L-citrulline, which is formed as a by-product of the NOS reaction, can be recycled to L-arginine by successive actions of argininosuccinate synthase and argininosuccinate lyase, forming the citrulline-NO cycle. On the other hand, arginase (isoformes type I. and II.) can down-regulate NO production by decreasing intracellular L-arginine concentrations. L-arginine is synthetized as a product of the urea cycle, circulates in the blood in concentrations of about 100 μM and is actively transported into the endothelium where its concentration is estimated to be several hundred to several thousand micromolars. The endothelium can even resynthesize this amino acid from L-citrulline in the absence of extracellular L-arginine [Harrison, 1997].

1.3. Interactions of estrogen and nitric oxide in gastroduodenal integrity

The sexual dimorphism of gastric or/and duodenal ulceration is well known. Clinical and experimental observations suggest that sexual steroids have a key importance in the regulation of the defensive mechanisms of the gastroduodenal mucosa [Robert and Kauffman, 1989; László et al., 1997; Drago et al., 1999; Szepes et al., 1999]. In earlier studies, gonadectomy decreased the severity of gastric mucosal injury in various models of ulceration [László et al., 1997; Drago et al., 1999]. It is also well established that numerous aggressive and protective factors affect the gastroduodenal mucosa, and an imbalance between them is pathogenic in the development of mucosal injury [Robert and Kauffman, 1989; Pávó et al., 2000]. Among the

protective factors, gastroduodenal mucus plays a crucial role [Robert and Kauffman, 1989]. Administration of NO donors stimulates mucus release from isolated gastric mucous-cell fraction (but not in parietal cells), and it has also been demonstrated that NOS is presented in these cells [Brown et al., 1992; 1993]. Moreover, an increase in endogenous NOS activity in the gastric mucosa protected the stomach against damage [Tepperman et al., 1993]. Inhibition of cNOS leads to microvascular dysfunction during the early-compensated phase of sepsis, at the initiation of bowel inflammation and in the course of surgical intervention [László et al.,1994; 1998; 1999]. Furthermore, a decreased cNOS activity and mucus level was found in the stomach in water immersion restraint stress-induced ulceration, effects reversed by NOS inhibition [Nishida et al., 1998, 1999].

1.4. The role of estrogen and nitric oxide in cardiovascular homeostasis

In the reproductive years, the incidence of ischemic heart disease among women is lower compared to men [Clarkson et al., 1997]. This gender difference can also be observed under experimental conditions, since it was shown that female rats were less prone to coronary artery occlusion-provoked ventricular arrhytmias compared to males [Humpreys et al., 1999]. The sexual dimorphism disappears after menopause; i.e. the incidence of coronary heart disease dramatically increases in women following their reproductive period to those levels that can be found in men of a similar age [Clarkson et al., 1997]. Moreover, the risk of ischemic heart disease is significantly higher among surgically postmenopausal women in comparison with premenopausal women in the same age [Kalin and Zumoff, 1990]. Population-based observational studies showed that estrogen replacement therapy in postmenopausal women reduced the mortality due to cardiovascular disease [Barrett-Connor and Bush, 1991]. It was also shown that estrogen replacement therapy increases endothelium-dependent relaxation in the coronary artery under experimental and clinical circumstances [Williams et al., 1992; Herrington, 1994]. These data suggest the cardiovascular protective role of endogenous and exogenous estrogen and it is suspected that the major site of the cardiovascular protective actions of estrogen is the arterial intima where most likely vascular endothelium-dependent mechanisms are involved.

A number of indirect *in vitro* (vascular endothelial cell culture, isolated organs, etc.) observations suggested that the liberation of NO from vascular tissues is regulated by sex hormones, e.g. estrogens were shown to enhance cNOS activity [Weiner et al., 1994]. This NO, formed continuously by cNOS in the vascular endothelium and neuronal elements, seems to play a significant protective role in the maintenance of vascular integrity, as the inhibition of cNOS provokes blood pressure elevation, platelet aggregation and adhesion of neutrophils to the vascular endothelium [Moncada and Higgs, 1995]. Moreover, NO donors, because of their smooth muscle relaxing effect, are well-known arterial and venous vasodilators. They inhibit neutrophil adherence to the vascular endothelium and platelet aggregation, processes that have crucial role in vasocongestion. It is also well established that NO donors are used world-wide as cardiac drugs, because of their direct coronary dilatory effects (high conductance big vessels), which putative mechanism plays an additional role in their protective action against myocardial ischemia [Rogers, 1996]. Thus, NO synthesized by cNOS preserves normal vascular tone, neutrophil deposition and platelet aggregation [Moncada and Higgs, 1993, 1995].

The selective estrogen-receptor modulators have tissue-specific estrogen agonist effects, e.g. on bone and lipid metabolism, and antagonist effects, e.g. on breast [Clarkson et al., 1997]. Indeed, increased NO release has been observed from the isolated rat aorta following the administration of selective oestrogen-receptor modulators such as raloxifene [Rahimian et al., 1997]. Furthermore, it has recently been demonstrated that estrogen can modulate the action of arginine-vasopressin on the baroreflex control of sympathetic outflow, and thereby participate in cardiovascular regulation [He et al., 1999].

2. OBJECTIVES

2.1. Estrogen and nitric oxide in gastroduodenal defence

In our gastrointestinal studies we aimed to examine the action of the estrogen-deficient state (i.e. ovariectomy) on gastric NOS activity, mucus secretion and on the susceptibility of the mucosa towards various ulcerogenic stimuli. We were also interested in the changes of cNOS and iNOS activity in conjunction with the possible role of endogenous bacteria in ovariectomy-provoked mucosal defence. We chose albumin leakage technique to monitor the effect of different ulcerogenic agents on the vascular permeability in the stomach.

2.2. Estrogen and nitric oxide in cardiovascular homeostasis

In parallel, we wished to investigate the actions of endogenous estrogens, directly on the regulation of cNOS enzyme activity under *in vivo* circumstances in the aorta and in the heart, obtained from the ovariectomized rat. Moreover, we aimed to measure the vasopressin-induced blood pressure response in catecholamine-depleted ovariectomized rats, and the effect of estrogen or raloxifene supplementation on this arginine-vasopressin provoked blood pressure response, in conjunction with the changes of aortic cNOS enzyme activity.

3. MATERIALS AND METHODS

3.1. Animals

Wistar rats (10-12 week-old) originated from Toxi Co., Budapest were used. Rats were housed 5 per cage in a room under constant conditions of illumination (12 hours light-dark cycle), temperature (22 ± 2°C) and humidity (20-25%). Standard diet and tap water were available *ad libitum*. After a week of habituation in the facilities, animals were admitted to the experimental sessions. All experiments were carried out according to the directives of the local Ethical Committee of the University and also of the European Communities Council Directive 86/609/EEC. Efforts were made to minimize animal suffering and to reduce the number of animals used.

3.2. Ovariectomy

Bilateral ovariectomy and sham-operation have been performed under ketamine and medetomidine (75 mg/kg and 0,5 mg/kg, respectively; i.p.) narcosis. Animals were allowed to recover over one month.

3.3. Gastric mucosal injury

For provocation of gastric mucosal injury, indomethacin (50 mg/kg, dissolved in absolute ethanol, s.c.) or cysteamine (400 mg/kg, dissolved in saline, s.c.) were administered, and the measurement of lesions has been performed 4 or 24 h later, respectively. The animals were fasted for 24 h before (indomethacin) or during (cysteamine) lesion-induction. Following autopsy, the extent of lesions in the stomach was determined by a digital planimeter (Placom KP-82N, Sokkia) and the ratio of injured parts was compared to the total mucosal surface, and the data were expressed as a per cent.

3.4. Vascular leakage

As an index of vascular endothelial damage, leakage of [^{125}I]human serum albumin was determined in the stomach. Under light ether anesthesia [^{125}I]human serum albumin (2 μCi/ kg) was administered into the tail vein 2 hours before autopsy. Immediately before autopsy (also under ether anesthesia) blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0,318%) and centrifuged (10000 g, 10 minutes, 4°C). The [^{125}I]human serum albumin content of the stomach and plasma was determined in a gamma spectrometer (Nuclear Enterprises NE1600). Control values (from rats that had received saline) were subtracted from the values of treated animals and data were expressed as changes in albumin accumulation (μl plasma/g tissue) as described previously [Boughton-Smith et al., 1993; László et al., 1999, Szepes 2001].

3.5. Gastric mucus level

The measurement of gastric mucus levels was performed using the method described by Corne et al. [Corne et al., 1974]. Before autopsy animals were fasted overnight, but received tap water *ad libitum*. After decapitation the glandular portion of the stomach were excised and immersed for 2 hours in 0,1% Alcyan blue in a 0.16 M/l sucrose solution buffered with 0.05 M/l sodium acetate (pH adjusted to 5.8 with HCl). The unbound dye was removed by two subsequent washing for 15 and 45 minutes in 0.25 M/l sucrose. The mucus-bound dye was eluted immersing the stomach in 0.5 M/l $MgCl_2$ solution for 2 hours. The optical density of the aqueous phase was measured at 605 nm in a spectrophotometer (Hitachi 150-20). The quantity of Alcyan blue, extracted per g of wet tissue, was then calculated from standard curves.

3.6. Measurement of nitric oxide synthase enzyme activity

The NOS enzyme activity was determined as the conversion of L-[^{14}C]-arginine monohydrochloride to L-[^{14}C]-citrulline based on the method described previously [Salter et al., 1991; Kiss et al., 2001, Whittle et al., 2001] with minor modifications aiming to detect

most sensitively the activity of cNOS [Weiner et al., 1994; Gross et al., 1991; Garvey et al., 1997; Morschl et al., 2000a]. We sacrificed the animals by decapitation, and immediately after autopsy, we prepared the fresh tissues for measurements. In different studies we examined the stomach (glandular portion), heart (the left ventricle from an individual rat) and aorta (pooled from the abdominal aorta of two rats). The tissues were homogenized (15 s, Ultra-Turrax homogenizer, 5 mm blade) in buffer (250 mg/ml, 4°C) containing 10 mM Hepes, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 2 μg/ml aprotinin at pH 7.4. Homogenates were centrifuged for 20 min (10000 g, 4°C). Supernatants were mixed with Dowex (AG 50W-8; 200-400, 8 % cross-linked, Na^+ form) resin and centrifugated for a further 10 min (10000 g, 4°C). Sample supernatant (40 μl) was incubated for 10 min at 37°C in reaction buffer comprising final concentrations of 50 mM KH_2PO_4, 10 μg/ml calmodulin, 2.5 mM $CaCl_2$, 50 mM valine, 1 mM dithiothreitol, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 μM FAD, 3 μM FMN, 3 μM THB_4 and 0.17 μM of [^{14}C]L-arginine. The reaction was arrested by the addition (0.5 ml) of a 1:1 v/v suspension of Dowex:water. After addition of 0.85 ml distilled water and settling for 30 min, the supernatant was removed for scintillation counting. Protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), and NOS activity was expressed as pM/min/mg protein. Total NOS activity was defined as citrulline formation that was abolished by incubation *in vitro* with N^G-nitro-L-arginine (L-NNA, 1 mM). Basal L-NNA-sensitive activity that was abolished by EGTA, was taken as calcium-dependent cNOS activity. In addition, calcium-independent NOS activity (iNOS) was also determined as the difference between samples containing 1 mM EGTA and samples containing 1 mM L-NNA.

3.7. Blood pressure response to vasopressin

Female rats were anaesthetised with urethane (1.25 g/kg, i.p.), and then pre-treated with phentolamine (10 mg/kg, i.p.). The elevation of blood pressure (expressed as a percent of the maximal increase compared to the basal value) was measured in the right carotid artery through a blood pressure transducer connected to a computerised complex haemodynamic analysis system (Haemosys, Experimetria U.K. Ltd., London). The core temperature of rats

was maintained at 37°C with a homeothermic control unit (Harvard Instrument, U.K.). When the blood pressure was stabilised, the effect of a single bolus injection of arginine-vasopressin (0.06-0.18 μg/kg) that was administered into the tail vein was measured.

3.8. Study groups and treatments

3.8.1. Gastroduodenal experiments

For the investigation of the role of NO in the secretion of gastric mucus, the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 10 mg/kg, s.c.) was administered into ovariectomized rats 4 h before any measurement.

To determine the role of endogenous bacteria in conjunction with the activation of iNOS, as a pre-treatment, groups of ovariectomized animals received the wide-spectrum antibiotic, ampicillin (800 mg/kg, p.o.) 48, 24 and 6 h before removal of the stomach, i.e. the latter 2 h before ulcerogenic challenge by indomethacin. Additional groups of ovary-intact female rats received exogenous bacterial endotoxin (*E. coli* lipopolysaccharide [LPS] 0111:B4, 3 mg/kg, i.v.) 5 h before autopsy, i.e. 1 h before indomethacin treatment, and than NOS activity and lesion-formation were measured.

Time-dependent changes of albumin leakage and the extent of mucosal lesions induced by indomethacin or cysteamine were examined simultaneously at 0.5, 1, 2 or 3, 6, 12 hours, respectively in the stomach of female rats.

Groups of ovariectomized animals received estrogen replacement therapy (17-β-estradiol, 20-100 μg/kg/day, s.c.) over a two-week period before cNOS activity measurement.

3.8.2. Cardiovascular experiments

Before cNOS activity measurement estrogen replacement therapy (17-β-estradiol, 20-100 μg/kg/day, s.c.) was introduced to groups of ovariectomized animals over a two-week period.

In the blood pressure response study, groups of ovariectomized rats received raloxifene (0.3-1 mg/kg, p.o., once daily) or 17β-oestradiol (0.3 mg/kg, p.o., once daily) for one month. The procedures, doses of compounds, and route of administration have been established in

previous studies [Rahimian et al., 1997; Morschl et al., 2000a]. Control animals received the vehicle at the same time, in the same volume and by the same route as the treated ones.

3.9. Chemicals and statistics

L-[^{14}C]arginine monohydrochloride, raloxifene, arginine-vasopressin and phentolamine were purchased from Amersham International (U.K.), Eli Lilly and Company (U.S.A.), Organon Oss (The Netherlands) and Ciba-Geigy (Switzerland), respectively. Alcyan-blue and urethane were obtained from Reanal (Hungary). [^{125}I]human serum albumin was obtained from Izinta, Budapest, Hungary. Ketamine and medetomidine (Domitor) were purchased from a commercial pharmacy. All other compounds were from the Sigma-Aldrich Chemical Company.

The data are expressed as the mean ± SEM of (n) rats per experimental group. Data were analyzed with one way ANOVA followed by the Tukey-Kramer multiple comparisons test, where $P<0.05$ was taken as significant.

4. RESULTS

4.1. Gastroduodenal experiments

4.1.1. Actions of ovariectomy following ulcerogenic stimuli

We found that after ovariectomy the severity of gastric lesions decreased by 63 ± 10 % or by 61 ± 6 % following indomethacin or cysteamine challenge, respectively (n=4-5, P<0.001), while NOS activity and mucus secretion increased (by 57 ± 8% and by 96 ± 11%, respectively, n=5-6, P<0.01). All of these effects of ovariectomy were reversed by the administration of the NOS inhibitor, L-NAME (Fig. 4.).

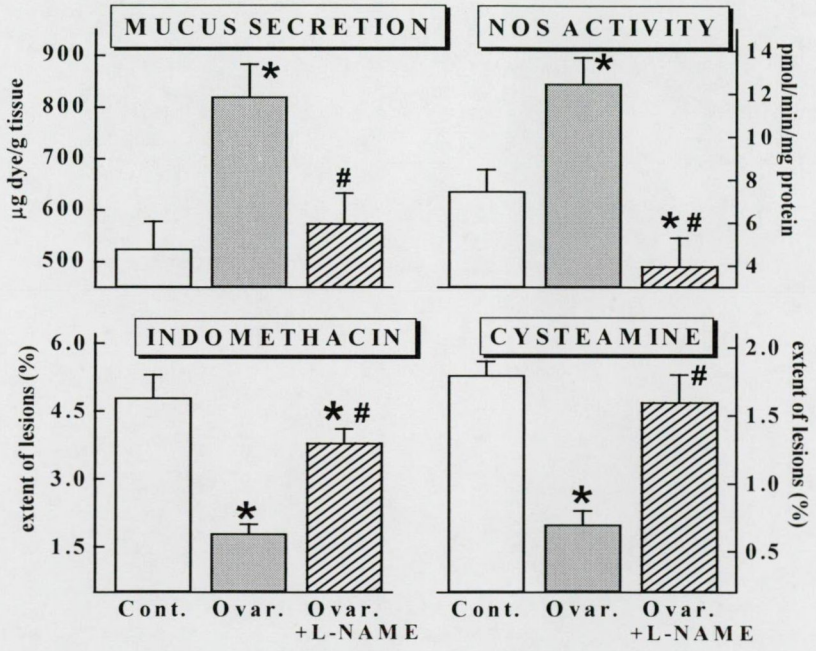

Fig. 4.

Actions of ovariectomy (Ovar., grey columns) on mucus secretion (expressed as µg dye/g tissue), NOS activity (expressed as pmol/min/mg protein) and lesion formation (expressed as extent of lesions in %) provoked by indomethacin or cysteamine in the stomach of the female rat (Cont., open columns), and their reversal by the administration of the NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, hatched columns). Data are expressed as mean ± S.E.M., where n=4-6 rats in a group. *P<0.05 between Cont. and Ovar. groups; $^{\#}$P<0.05 between Ovar. and Ovar.+L-NAME groups.

4.1.2. Effects of ovariectomy on nitric oxide synthase enzyme activity

The total NOS activity was enhanced in the ovariectomized rat stomach, which originated from the changes of cNOS and iNOS activities. We found a mild, but significant decrease in cNOS activity (by 31 ± 8 %; n=18; $p<0.05$), and a profound increase in iNOS activity (by 719 ± 10 %; n=10; $p<0.001$) as shown in Fig. 5.

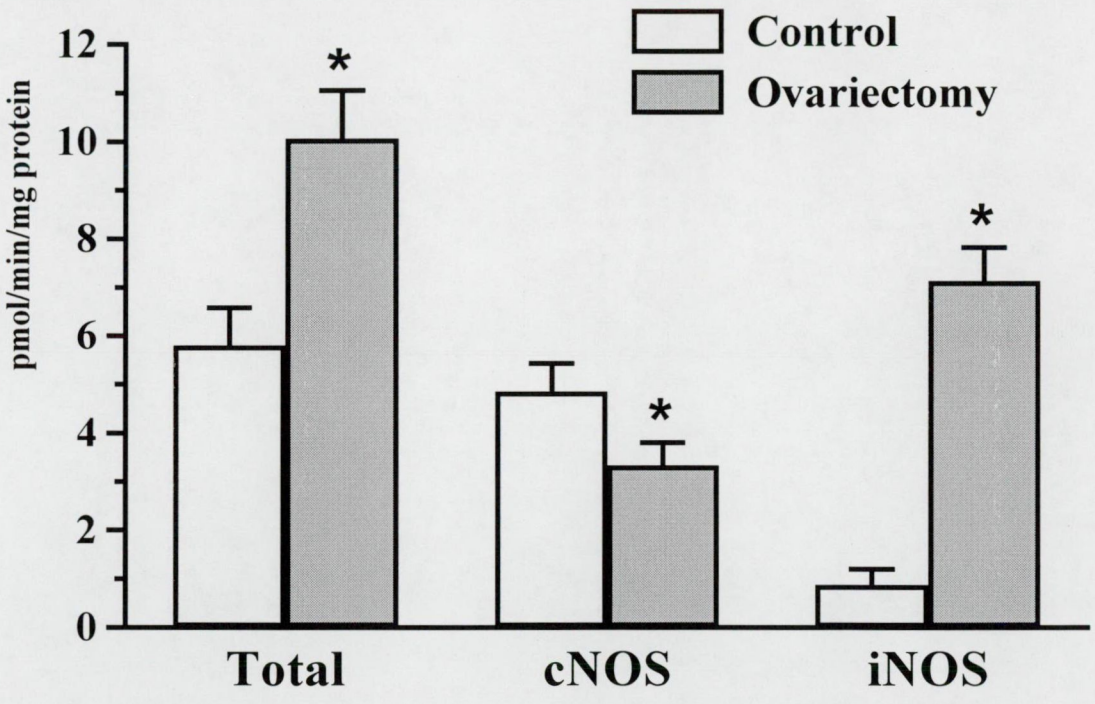

Fig. 5.

Total, constitutive and inducible NOS (cNOS and iNOS, respectively) activity (assessed by the citrulline assay, and expressed as pmol/min/mg protein) in the stomach of control (white column) and ovariectomized (gray column) female rats. Data are shown as the mean ± S.E.M., where (n) is at least 10 measurements in a group, and where statistical significance is given as *P<0.05 between ovariectomized and control groups.

4.1.3. The effect of ampicillin on the actions of ovariectomy

The pre-treatment with ampicillin reversed ovariectomy-provoked gastric mucosal defense after indomethacin challenge (by 94 ± 11 %, n=5, $P<0.05$), and abolished the increase in iNOS activity (by 99 ± 1 %, n=5, $P<0.001$) in the ovariectomized rat stomach. Data are demonstrated in Fig. 6. During this study, no change in cNOS activity could be observed (data are not shown).

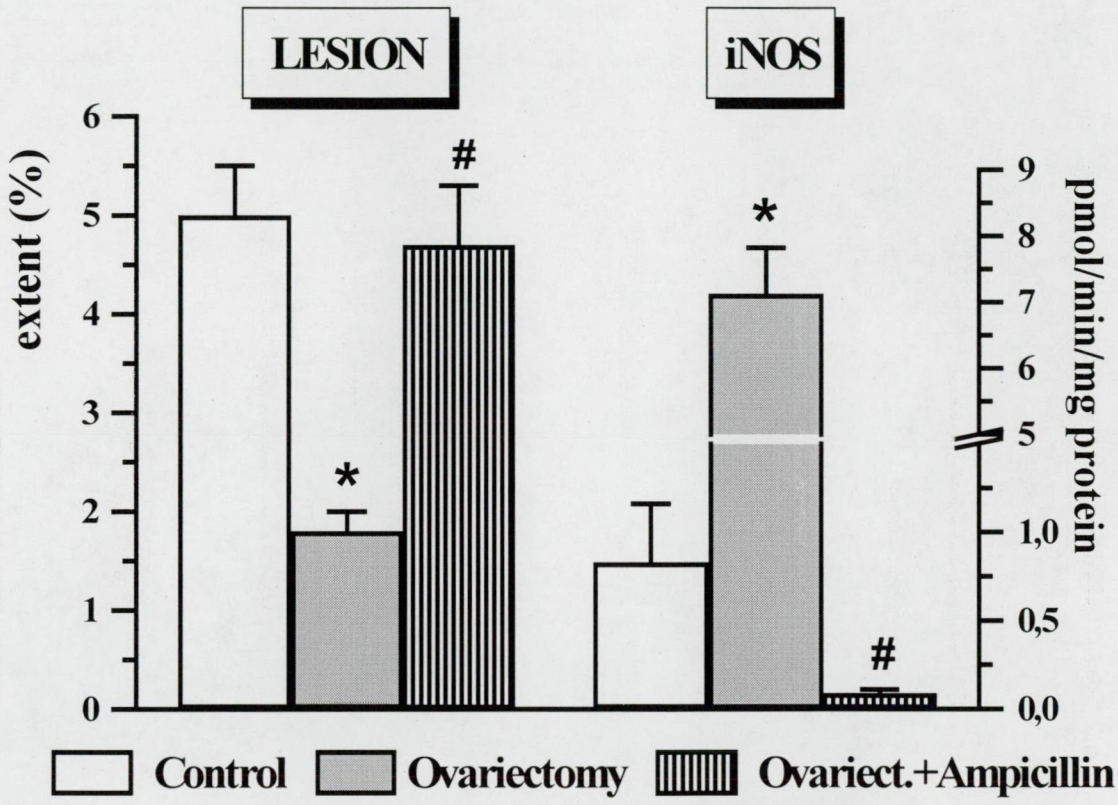

Fig. 6.

Indomethacin (50 mg/kg, s.c., 4h)-induced lesion formation (expressed as the % of the total gastric mucosal surface), and iNOS activity (expressed as pmol/min/mg protein) in the stomach of control (ovary-intact), ovariectomized (ovariect.) and ampicillin-treated (800 mg/kg, p.o., 3 days) ovariectomized female rats. Data are shown as the mean ± S.E.M., where (n) is at least 5 rats in a group, and where statistical significance is given as *$P<0.001$ between ovariectomized and control groups; $^{\#}P<0.05$ between ampicillin-treated ovariectomized and ovariectomized groups.

4.1.4. The effect of endotoxin on the actions of ovariectomy

Administration of *E. coli* endotoxin increased the activity of iNOS (by 491 ± 6 %, n=5, $p<0.001$) and decreased indomethacin-induced lesion-formation (by 68 ± 7 %, n=5, $p<0.01$) in the stomach of ovary-intact female rats. Data are demonstrated in Fig. 7. We found that cNOS activity did not change throughout this experimental series (data are not shown).

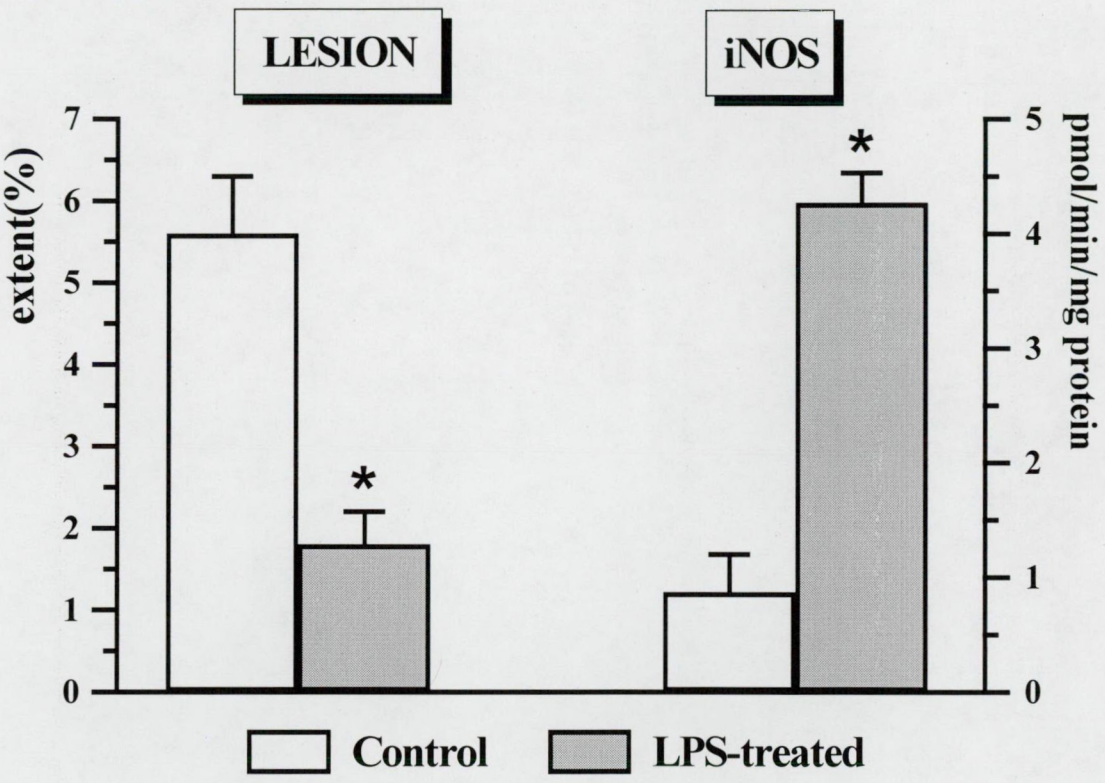

Fig. 7.

Indomethacin (50 mg/kg, s.c., 4 h before autopsy)-induced lesion formation (expressed as the % of the total gastric mucosal surface), and iNOS activity (expressed as pmol/min/mg protein) in the stomach of control (ovary-intact) female rats, and the effects of the administration of exogenous bacterial endotoxin (LPS; *E. coli* 0111:B4, 3 mg/kg, i.v., 5 h before autopsy). Data are shown as the mean ± S.E.M., where (n) is at least 5 rats in a group, and where statistical significance is given as *$P<0.001$ between LPS-treated and control groups.

4.1.5. Vascular leakage following ulcerogenic stimuli

Thirty minutes following indomethacin administration the albumin leakage was significantly high (26 ± 4 µl/mg), showing that vascular permeability increased, while the extent of mucosal lesions became significant (1.28 ± 0.14 %) only after 1 hour. Both albumin leakage and the extent of lesions expanded in a time-dependent manner. In the cysteamine treated group the extent of lesions became significant after 3 hours (0.6 ± 0.1 %) and expanded with time, but albumin leakage was not significant until 12 hours (47 ± 3 µl/mg). Data are shown in Fig. 8.

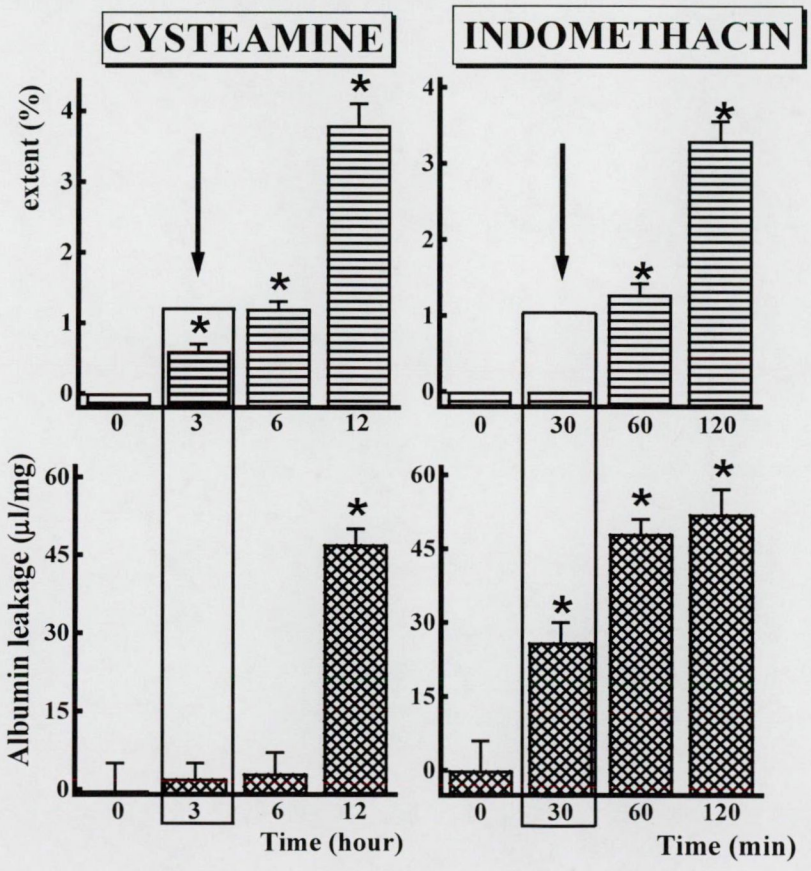

Fig. 8.

Time dependent changes in mucosal lesions (extent of lesions, as the percent of the total mucosal surface) and vascular permeability (albumin leakage, expressed as µl albumin/g tissue) after indomethacin and cysteamine treatment in the stomach of female rats. Data are shown as mean ± S.E.M., where n=6-8, and statistical significance is shown as *P<0.01 compared to the basal (0 hour or 0 min) values.

4.2. Cardiovascular experiments

4.2.1. The effect of estrogen on nitric oxide synthase enzyme activity

We found that cNOS activity was higher in the aorta and heart of female rats compared to males (105 ± 10 % and 104 ± 18 %, respectively; n=7-9; $p<0.001$). Ovariectomy decreased cNOS activity in both tissues (by 58 ± 6 % in the aorta and by 49 ± 4 % in the heart; n=7-9; $p<0.001$) to that level what could be observed in males. Estrogen supplementation in the ovariectomized rat caused a dose-dependent elevation of cNOS enzyme activity in cardiac and aortic tissues, where the higher dose (100 μg) completely restored cNOS enzyme activity to that level what could be observed in females. Data are shown in Fig. 9.

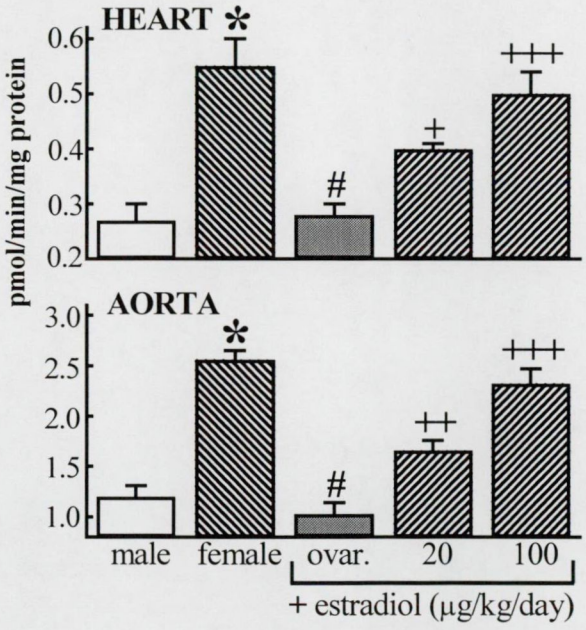

Fig. 9.

Actions of a two-week estrogen replacement therapy (17-β-estradiol, 20-100 μg/kg/day, s.c.) on the activitiy of cNOS (citrulline assay, expressed as pmol/min/mg protein) in the left ventricle of the heart and abdominal aorta of the male, female and ovariectomized (ovar.) female rat. Data are shown as the mean ± S.E.M., where (n) is at least 5 rat in a group. Statistical significance is given as 1./ *$P<0.001$ between male and female groups, 2./ $^{\#}P<0.001$ between female and ovariectomized female groups, 3./ $^{+}P<0.05$, $^{++}P<0.01$, $^{+++}P<0.001$ between ovariectomized female rats and those ovariectomized animals that received estrogen replacement therapy.

In female and male rats, there was no significant difference in iNOS activity in the aorta (being 0.07 ± 0.04 and 0.09 ± 0.04 pmol/min/mg protein, respectively; n=7-8) and in the heart (being 0.25 ± 0.06 and 0.22 ± 0.05 pmol/min/mg protein, respectively; n=7-8) under basal conditions. Neither ovariectomy nor estrogen treatment caused change in aortic and cardiac iNOS activities compared to the appropriate basal values (n=7-9, data are not shown).

4.2.2. The effect of raloxifen on nitric oxide synthase enzyme activity

The decreased cNOS activity in the aorta of the ovariectomized rat could be restored by both 17β-estradiol (0.3 mg/kg) or raloxifene (1 mg/kg) supplementation (1 month, p.o.) to levels found in intact females (Fig.10.).

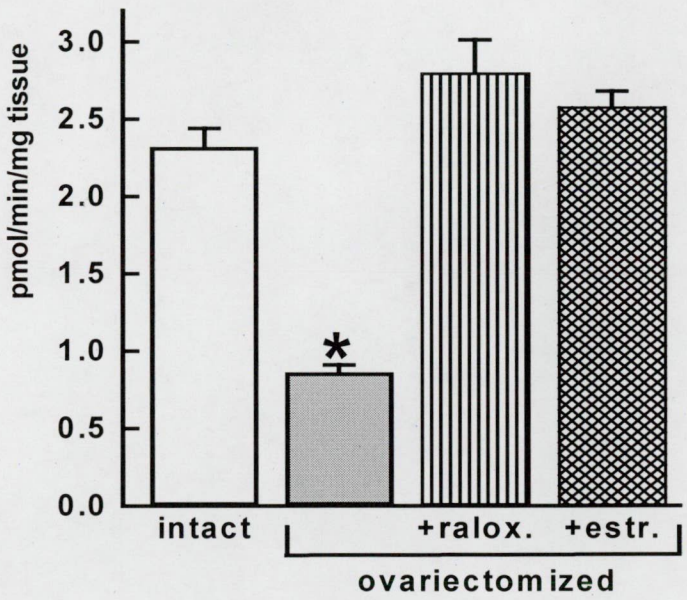

Fig. 10.

Actions of raloxifene (ralox.; 1 mg/kg) or 17β-estradiol (estr.; 0.3 mg/kg) supplementation (1 month, p.o.) on cNOS enzyme activity (citrulline assay, expressed as pmol/min/mg tissue) in the aorta of ovariectomized rats. Data are expressed as mean ± S.E.M. of 5 measurements in each group; *P<0.001 means significant difference between the intact female and the ovariectomized group.

Neither ovariectomy nor administration of 17β-estradiol or raloxifene changed aortic iNOS activities compared to the intact basal values (n=5, data not shown).

4.2.3. The effect of estrogens on blood pressure

Administration of arginine-vasopressin caused a dose-dependent increase of arterial blood pressure both in the ovary-intact and ovariectomized female rat. However, in the ovariectomized animals, vasopressin induced a significantly higher elevation of blood pressure than in ovary-intact females. Estrogen replacement abolished the increased blood pressure response observed, and raloxifene supplementation caused a dose-dependent decrease in blood pressure enhancement provoked by arginine-vasopressin in ovariectomized rats (Fig.11.).

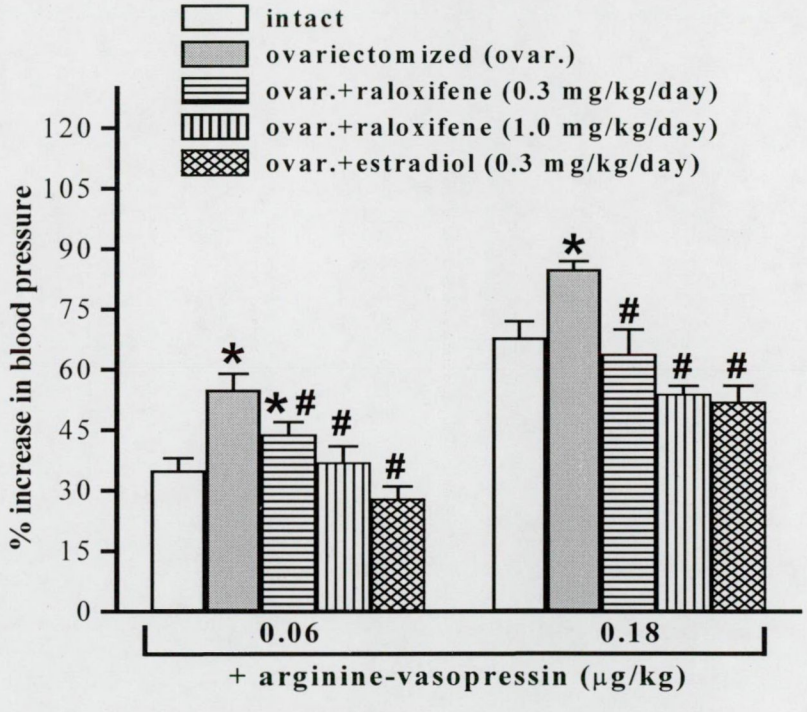

Fig. 11.

Increase of arterial blood pressure (expressed as % change of basal value, measured in the right carotid artery) by intravenous administration of arginine-vasopressin (0.06-0.18 μg/kg) in the catecholamine-depleted (phentolamine, 10 mg/kg, i.p.) sham-operated and ovariectomized (1 month) female rat. Effect of supplementation (1 month, p.o.) with 17β-estradiol (0.3 mg/kg/day) or the selective estrogen-receptor modulator, raloxifene (0.3-1 mg/kg/day) on arginine-vasopressin induced blood pressure elevation in the ovariectomized rat. Data are expressed as mean ± S.E.M., where n=8-12 rats in a group. *$P<0.05$ means significant blood pressure increase compared to the arginine-vasopressin treated intact female group; $^{\#}P<0.05$ means significant blood pressure decrease compared to the arginine-vasopressin treated ovariectomized female group.

5. DISCUSSION

5.1. Gastroduodenal experiments

5.1.1. Actions of estrogen following ulcerogenic stimuli

Our findings confirm that gonadectomy improves the defensive mechanism of the stomach [László et al., 1997; Drago et al., 1999; Szepes et al., 1999]. It seems that female sexual steroids have dual actions on the gastric mucosa. Progesterone prevents injury, since during early pregnancy, when progesterone level is high, a lower susceptibility of the stomach has been found [Montoneri and Drago, 1997]. In contrast, estrogens augment damage, because of 1./ the gastric protection by ovariectomy and lactation, when the level of estrogens is low [Robert and Kauffman, 1989; Drago et al.; 1999], and 2./ the increased generation of gastric lesions following the exogenous administration of high doses of estrogen [Szepes et al., 1999]. These observations are apparently conflicting with the well-known experience i.e. males are more prone to gastric ulceration than females, although, the estrogen level in males is known to be low. However, in males, orchidectomy or testosterone antagonist revealed to protect against injury, which show that in males, instead of estrogens, testosterone plays an aggressive role towards the gastric mucosa [László et al., 1997]. In addition, testosterone may generate more severe gastric damage in males compared to females, since in males the protective progesterone level is also low.

5.1.2. Mechanisms of estrogen-mediated mucosal defence

In our studies, increased gastric mucosal NOS activity has been shown after ovariectomy. This elevated gastric NO production might have important role in the protection against ulcerogenic challenge, since the administration of the NOS inhibitor, L-NAME restored mucosal damage in the ovariectomized rat to that level what could be observed in control females.

The increased generation of NO may play a role among gastric protective mechanisms by its ability to maintain microvascular integrity [Whittle, 1993, 1995] which is known to be an

important factor in the development of mucosal injury [Robert and Kauffman, 1989]. In the case of indomethacin-induced gastric mucosal injury this NO-mediated microcirculatory mechanism is more likely to be involved, since in this model vascular factors are known to have crucial pathogenic role [Robert and Kauffman, 1989; Whittle, 1993]. Indeed, in our time-response study, we found that microvascular injury preceded mucosal damage following indomethacin administration, while the opposite could be observed with cysteamine.

We determined that in ovariectomized rats the mucus secretion in the stomach has been increased, which could be reversed by L-NAME administration. Thus, NO mediates mucus overproduction in the ovariectomized rat. This mucus hypersecretion may be the common pathway, which explains why the estrogen-deficient state protects the gastric mucosa against different ulcerogenic stimuli, i.e. following indomethacin or cysteamine administration.

Our results showed that the elevation of iNOS activity seems to be responsible for the significant increase of total NOS activity in estrogen-deficient rats, since in the same time, we found cNOS activity to be decreased, which corresponds our findings in the heart and aorta [Morschl et al., 2000]. Thus, estrogen-deficiency down-regulates cNOS in the stomach, too, similarly that found by others in the vascular tissue [Weiner et al., 1994; Rahimian et al., 1997]. The decrease of physiological cNOS activity is known to initiate an increase in neutrofil infiltration to the vascular endothelium, platelet aggregation, impaired vasodilatation and elevation of vascular permeability, processes, which attenuate blood supply towards the gastric tissue [Moncada and Higgs, 1995; Whittle, 1993]. Moreover, a reduction in cNOS activity decreases mucus level in the stomach [Nishida et al., 1998; Morschl et al., 2000]. All these processes attenuate gastric defence mechanisms.

It is strongly suspected that this weaker defensive state makes the mucosa more susceptible for the penetration of endogenous bacteria towards the deeper layers of gastric tissues where they may trigger iNOS activation. Indeed, administration of the wide spectrum antibiotic ampicillin diminished the expression of iNOS, and abolished the gastric mucosal defense provided by estrogen-deficiency, i.e. by ovariectomy [Morschl et al., 2001]. In an additional series of studies, we also showed that administration of exogenous bacterial endotoxin protected the gastric mucosa against indomethacin-induced ulceration and increased iNOS activity in ovary-intact rats confirming earlier observations, where different ulcer models were used [Tsuji et al., 1993; Yu et al., 1997]. Moreover, other investigators

demonstrated that an enhanced generation of endogenous NO caused by neutrophil depletion also improved the defensive mechanisms of the stomach [Tepperman et al., 1993]. Finally, NO, administered exogenously using NO donors, significantly ameliorated gastric mucosal damage provoked by various ulcerogenic agents [Lopez-Belmonte et al., 1993; Mourad et al., 2000]. Thus, a controlled elevation in gastric NO level, originated from an increased endogenous NO production or exogenous NO administration, defends the stomach against injury. The above-described findings give further support for the protective role of increased NOS enzyme activity by the expression of iNOS, and, in addition, suggests that the endotoxin component of endogenous gram-negative bacteria can be responsible for iNOS activation resulting gastric mucosal protection in the estrogen-deficient state, i.e. following ovariectomy.

5.2. Cardiovascular experiments

5.2.1. Effects of estrogen on nitric oxide synthase enzyme activity

We found that the activity of the cNOS enzyme in cardiac and aortic tissues is lower in the estrogen-deficient state (i.e. male and ovariectomized female rat). Our observations are in agreement with previous findings where isolated aortic rings of female rabbits, mice and rats were shown to release greater amount of NO than ovariectomized females or male animals [Hayashi et al., 1992; Kauser and Rubányi, 1992; Rubányi et al., 1997]. Moreover, in human vascular endothelial cell culture, estrogen increased cNOS activity via a receptor-mediated system [Hayashi et al., 1997]. In previous studies, it has been demonstrated that estrogen treatment of cultured endothelial cells enhanced calcium-dependent NO production and eNOS protein synthesis [Hishikawa et al., 1995], and estrogen replacement therapy following gonadectomy increased eNOS mRNA expression in the rat aorta [Goetz et al., 1994]. Therefore, it is strongly suspected that the enhanced cNOS activity following estrogen treatment reflects an increased physiologically available NO production. We demonstrated that the administration of graded doses of exogenous estrogen increased and even restored cNOS enzyme activity in cardiac and aortic tissues of the ovariectomized rat. Indeed, a reduced vascular tone has been found during pregnancy when the plasma concentration of estrogen is high [Everson, 1992], and administration of estradiol increased endothelium-

dependent vascular relaxations [Miller and Vanhoutte, 1991]. Our results accord with earlier *in vitro* observations, where physiological levels of circulating 17-β-estradiol has been shown to elevate basal NO release from endothelial cells, which amount of NO was sufficient to increase the diameter of pressurized coronary arteries in rats [Wellman et al., 1996]. Our results are also in agreement with the findings of Weiner and his co-workers, the increase of endogenous estrogen level (by pregnancy) or administration of exogenous estrogen to intact females elevated cNOS enzyme activity and mRNA expression in the uterine artery and heart of guinea pigs [Weiner et al., 1994]. In a recent report, it has been demonstrated that estrogen administration provokes the expression of eNOS protein in rat cardiac myocytes both under *in vivo* and *in vitro* circumstances [Nuedling et al., 1999], which results may provide a further insight for the better understanding of estrogen-mediated cardiac protection. Moreover, increased amount of myocardial NO by the activation of iNOS following pacing, exercise and endotoxin treatment also causes a marked reduction in sudden cardiac death following coronary artery occlusion in various species [Kiss et al., 1999ab; Végh et al., 1992, 1999]. In addition, estradiol supplementation improved vasodilatation in postmenopausal women, because of augmented NO production [Kawano et al., 1997]. Finally, chronically administered estrogen enhanced the release of NO from the vascular endothelium of the rat aortic ring [Rahimian et al., 1997].

In a large number of studies, it has been demonstrated that NO synthesized by cNOS has a key beneficial importance in the maintenance of vascular integrity. It is known that inhibition of cNOS increases blood pressure, platelet aggregation and neutrophil adherence to the vascular endothelium. On the basis of numerous observations, it is strongly believed that NO synthesized in the vascular endothelium (eNOS) plays the crucial role in the beneficial vascular actions of cNOS [Moncada and Higgs, 1993, 1995]. However, more and more recent data are cumulating that NO synthesized in neuronal elements (nNOS) has similar vascular protective actions [Weiner et al., 1994; Moncada and Higgs, 1995] which activity is also included in cNOS. It seems that further studies are needed to clarify the role of nNOS in cardiovascular protection.

5.2.2. Effects of estrogen on blood pressure response

The findings of our blood pressure response study are in agreement with recent observations that vasoconstrictor tone is increased in various vascular beds following ovariectomy, an effect that can be mitigated by estrogen or raloxifene therapy [He et al., 1999; Zoma et al., 2000]. This impaired vasodilatation most likely originates from the down-regulation of cNOS in the estrogen-deficient state. Under *in vitro* circumstances, a reduction of the basal release of NO, as detected by augmented catecholamine-induced contractile response in the presence of a NOS inhibitor, was found in aortic rings from ovariectomized animals, an effect blocked by raloxifene or estradiol treatment [Rahimian et al., 1997]. Under *in vivo* conditions, we found a reduced cNOS activity in the aortic tissue of ovariectomized rats compared with normal females, and administration of 17β-estradiol dose-dependently increased aortic cNOS activity in ovariectomized rats up to normal female levels. These results give further support to the proposition that vascular cNOS activity is regulated by estrogen, and that under *in vivo* circumstances raloxifene has estrogen agonist properties with respect of the regulation of the vascular cNOS enzyme.

There are pathological circumstances in which release of NO by cNOS from an intact endothelium protects the vascular tissue against the potentially injurious effects of endogenous vasoconstrictors such as arginine-vasopressin [Pávó et al., 2000; László et al., 2001]. For example, in the early compensated phase of endotoxaemia, severe vascular dysfunction can occur following endotoxin challenge when cNOS is inhibited, whereas neither endotoxin nor NOS inhibitor alone cause dysfunction, and this injury is reversed by a vasopressin antagonist [László and Whittle, 1994]. Moreover, in the operating theatre, cNOS has been demonstrated to maintain vascular integrity [László and Whittle, 1999; László et al., 1999; Pávó et al., 2000]. Constitutive NO effectively counteracts the increase in vascular permeability provoked by arginine-vasopressin which is known to be released during major surgical operations [Melville et al., 1985; Pávó et al., 2000]. Thus, in the ovariectomized state, impaired expression or/and activity of cNOS may explain the increased blood pressure response to arginine-vasopressin in the absence of estrogen.

6. SUMMARY

6.1. The role of estrogen and nitric oxide in gastroduodenal defence

We suppose that gastric protection against ulcerogenic stimuli following ovariectomy is mediated by an increase in total NOS enzyme activity. Although cNOS activity was significantly decreased, iNOS activity was highly elevated in the stomach resulting in a significant elevation of total NOS. We demonstrated that in the estrogen-deficient state, endogenous bacteria trigger the activation of iNOS, and the increased total NO production by iNOS expression leads to an increased gastric mucus secretion, which improves gastric mucosal defence against ulcerogenic challenge.

6.2. The role of estrogen and nitric oxide in cardiovascular homeostasis

Our results showed that estrogen-deficiency down-regulates aortic cNOS. The diminished generation of NO by cNOS seems to be involved in the increased sensitivity of the vasculature to the vasoconstrictor effect of arginine-vasopressin, since both actions can be reversed by natural estrogen (17β-estradiol) or selective estrogen-receptor modulator (raloxifene) therapy. Thus, raloxifene behaves as an estrogen receptor agonist with regards to both regulations of cardiovascular cNOS and arginine-vasopressin provoked increases in blood pressure *in vivo*. The findings of our studies suggest that endogenous estrogen up-regulate cNOS enzyme in the rat aorta and heart. This estrogen-mediated increase of NO production by cNOS might possibly be an explanation of the sexual dimorphism of ischaemic heart disease, i.e. women are less sensitive than men in the fertile age.

7. PERSPECTIVES

On the basis of our present experimental observations and the findings of other investigators, it seems that selective estrogen-receptor modulators might have potential therapeutic benefit in the treatment or/and prevention of ischemic heart disease. Because these compounds do not have estrogen agonist properties towards the breast and genital tissues, however they behave as estrogen-receptor agonists towards the bone metabolism, the blood lipid profile and the cardiovascular system, selective estrogen-receptor modulators (such as raloxifene) would open new therapeutic perspectives of human coronary heart disease. We showed that endogenous and exogenous estrogen, and selective estrogen-receptor modulator up-regulate cardiac and vascular cNOS. The increased generation of NO by estrogen may protect the heart better than the conventionally used nitrovasodilators, since it is strongly suspected that nitrate tolerance does not occur following chronic estrogen-receptor modulator treatment compered to nitrovasodilators where the development of nitrate tolerance attenuates their clinical efficacy. Thus, it seems that in human estrogen deficiency, i.e. in men and in postmenopausal women, selective estrogen-receptor modulators could be a good additional therapeutic choice of treating ischemic heart disease.

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