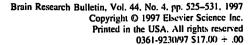
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# Role of Astroglia and Insulin-Like Growth Factor-I in Gonadal Hormone-Dependent Synaptic Plasticity

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ABSTRACT: Gonadal hormones exert a critical influence over the architecture of specific brain areas affecting the formation of neuronal contacts. Cellular mechanisms mediating gonadal hormone actions on synapses have been studied extensively in the rat arcuate nucleus, a hypothalamic center involved in the feed-back regulation of gonadotropins. Gonadal steroids exert organizational and activational effects on arcuate nucleus synaptic connectivity. Perinatal testosterone induces a sexual dimorphic pattern of synaptic contacts. Furthermore, during the preovulatory and ovulatory phases of the estrous cycle there is a transient disconnection of inhibitory synaptic inputs to the somas of arcuate neurons. This synaptic remodeling is induced by estradiol, blocked by progesterone, and begins with the onset of puberty in females. Astroglia appear to play a significant role in the organizational and the activational hormone effects on neuronal connectivity by regulating the amount of neuronal membrane available for the formation of synaptic contacts and by releasing soluble factors, such as insulin-like growth factor I (IGF-I), which promote the differentiation of neural processes. Recent evidence indicates that gonadal steroids and IGF-I may interact in their trophic effects on the neuroendocrine hypothalamus. Estradiol and IGF-I promote the survival and morphological differentiation of rat hypothalamic neurons in primary cultures. The effect of estradiol depends on IGF-I, while the effects of both estradiol and IGF-I depend on estrogen receptors. Furthermore, estrogen activation of astroglia in hypothalamic tissue fragments depends on IGF-I receptors. These findings indicate that IGF-I may mediate some of the developmental and activational effects of gonadal steroids on the brain and suggest that IGF-I may activate the estrogen receptor to induce its neurotrophic effects on hypothalamic cells. In addition, IGF-I levels in the neuroendocrine hypothalamus are regulated by gonadal steroids. IGF-I levels in tanycytes, a specific astroglia cell type present in the arcuate nucleus and median eminence, increase at puberty, are affected by neonatal androgen levels, show sex differences, and fluctuate in accordance to the natural variations in plasma levels of ovarian steroids that are associated with the estrous cycle. These changes appear to be mediated by hormonal regulation of IGF-I uptake from blood or cerebrospinal fluid by tanycytes. These results suggest that tanycytes may be involved in the regulation of neuroendocrine events in adult rats by regulating the availability of IGF-I to hypothalamic neurons. In summary, IGF-I and different forms of neuron-astroglia communication are involved in the effects of estradiol on synaptic plasticity in the hypothalamic arcuate nucleus. © 1997 Elsevier Science Inc.

KEY WORDS: Insulin-like growth factor-I, Estradiol, Estrogen receptors, Glia, Astrocytes, Tanycytes, Synpatic plasticity.

#### INTRODUCTION

Gonadal hormones exert profound influences on neural development and function, mainly, although not exclusively, in brain areas related to neuroendocrine control and sexual behavior. Sex steroids exert organizational effects on brain development and activational effects on the adult brain. Indeed, the sexual dimorphism that exists in the adult brain is induced by androgen effects on neuronal differentiation, with part of these effects being subsequent to the intracerebral aromatization of androgens to estrogens [50]. Estradiol promotes the survival of neurons and the growth of neuronal processes, regulating the formation of neuronal connectivity [20,43], and therefore, the function of neuronal networks. Furthermore, recent evidence indicates that glial cells are affected by gonadal steroids and are actively involved in the organizational and activational effects of these hormones [9,19,27,40,44,57].

Cellular mechanisms mediating gonadal hormone actions on synapses and glial cells have been extensively studied in the rat arcuate nucleus, a hypothalamic center involved in the feed-back regulation of gonadotropins. Perinatal androgens induce the sexual differentiation of synaptic connectivity in the arcuate nucleus [21,43]. Furthermore, in postpuberal females, there is an estrogeninduced transient disconnection of GABAergic inputs to the somas of arcuate neurons during the preovulatory and ovulatory phases of the estrous cycle. This synaptic remodelling is blocked by progesterone and begins with the onset of female puberty [20]. Astrocytes appear to play a significant role in these synaptic changes [20,22]. In the afternoon of proestrus, estradiol induces synthesis of glial fibrillary acidic protein (GFAP), a specific component of astroglia cytoskeleton [20,22,34]. Synthesis of GFAP is accompanied by the growth of glial processes that ensheathe the neuronal membrane and displace the synaptic terminals. Glial processes retract and synapses reform in the afternoon of estrus [20,22]. Glial cells also appear to be involved in the organizational effects of sex steroids on arcuate neurons [9,21]. Testosterone appears to regulate the number of synaptic inputs to arcuate neurons by affecting the amount of neuronal membrane covered by glial processes and. therefore, the amount of membrane available for the formation of synaptic contacts [21].

The signaling mechanisms involved in these hormonal ef-

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fects on neurons and glia have been investigated in hypothalamic cultures. The estradiol-induced growth of astrocyte processes in vitro is dependent on the preexisting direct contact of glial cells with living neurons and on specific neuron-astrocyte interactions, because it is not observed in cocultures of hypothalamic neurons with astrocytes from other brain areas [18,20]. The expression of a polysialic acid-enriched embryonic form of the neural cell adhesion molecule in the neuronal membranes is essential for the glial plastic changes induced by estradiol, but not for those induced by signals such as fibroblast growth factor that act directly on astrocytes [18,20]. This finding suggests that a neuron to astrocyte signaling pathway participates in the plastic effects of gonadal hormones. In addition, glial cells release different soluble factors that alter neuronal function and that may be involved in the regulation of synaptic connectivity and neuroendocrine function [11,40,46]. One such factors is insulin-like growth factor-I (IGF-I), a cytokine with paracrine trophic effects on neural cells and that acts as a hormonal signal involved in the regulation of hypothalamic hormone secretion. Here, we review recent evidence indicating that IGF-I is involved in some of the effects induced by gonadal hormones on hypothalamic neurons.

### IGF-I AND ESTROGEN SIGNALING INTERACT IN DEVELOPING HYPOTHALAMIC NEURONS

IGF-I is locally synthesized by glia and neurons of the hypothalamus as well as of other brain areas [5,23] and has prominent trophic actions, stimulating survival, proliferation, and differentiation of specific neural cell populations [37,63]. IGF-I may also participate in neuroendocrine events at the level of the hypothalamus because it has been shown to be involved in the feed-back regulation of growth hormone by affecting the synthesis or the release of growth hormone-releasing hormone and somatostatin by hypothalamic neurons [3,60]. IGF-I may also affect the reproductive axis by modulating the release of gonadotrophin releasing hormone by hypothalamic cells and, therefore, the release of gonadotrophins [6,25].

In several tissues, such as rodent uterus and pituitary, and in several cell types, such as human breast cancer cells, estrogen upregulates IGF-I gene expression [17,28,47,49,58] and modulates IGF-I action by affecting the levels of IGF-I receptors [67] and IGF binding proteins (IGFBPs) [36,48,53,69]. Likewise, IGF-I may regulate steroid hormone action by stimulating the synthesis of steroid hormones [10,13,24] and steroid hormone receptors [1,8,31]. In addition, estrogen and antiestrogens regulate several cellular responses induced by IGF-I [1,16,61,68]. As in other cell types, IGF-I and estrogen may also have interactive effects on neurons. The first evidence was provided by Toran-Allerand and co-workers [62], showing that in explant cultures of fetal rodent hypothalamus, estrogen and insulin have synergistic effects on neurite growth, an effect probably mediated by IGF-I receptors. More recent data from our laboratory indicates that estrogen modulates IGF-I receptors and binding proteins in monolayer hypothalamic cultures [56].

We have also recently studied whether the effects of estrogen on hypothalamic neuronal survival and neurite growth are mediated by IGF-I [12]. To assess the effect of  $17\beta$ -estradiol and IGF-I on the survival and differentiation of hypothalamic neurons, the hormone and/or peptide were added to rat hypothalamic monolayer cultures every 2 days for 6 days at a final concentration of  $10^{-9}$  M. Neuronal survival and growth of microtubule associated protein-2 (MAP-2) immunoreactive processes was then assessed. Neurons exposed to estradiol or IGF-I showed a significant increase in neuronal survival and in

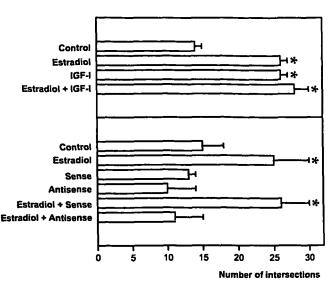


FIG. 1. Extension of microtubule associated protein-2 (MAP-2) Immunoreactive processes from hypothalamic neurons in monolayer culture. The extension of MAP-2 immunoreactive processes was evaluated by counting their intersections with a morphometric grid (see [12]). Both IGF-1 (10 "M) and estradiol (10 -9 M), either alone or in combination, induced the growth of MAP-2 immunoreactive processes (upper panel). Inhibition of IGF-1 synthesis by incubating the cultures with an antisense oligonucleotide to IGF-1 (33  $\mu$ g/ml) resulted in the inhibition of the effect of estradiol (lower panel). A sense control sequence was without effect. Asterisks indicate significant differences (p < 0.01) vs cultures incubated in control medium.

the growth of MAP-2 immunoreactive processes (Fig. 1). The simultaneous incubation of the cultures with  $17\beta$ -estradiol and IGF-I also resulted in increased neuronal survival and differentiation. The effect of estradiol and IGF-I acting together was similar to that observed when cultures were treated separately with either factor (Fig. 1). Therefore, the effects of  $17\beta$ -estradiol and IGF-I were not additive, suggesting that both factors may be acting through a common mechanism. We decided. therefore, to test whether the effect of estradiol was dependent on IGF-I synthesis. The cultures were thus exposed for 2 days to an antisense oligonucleotide to IGF-I [12]. Incubation of the cultures with the antisense sequence resulted in a significant decrease in the stimulatory effects of  $17\beta$ -estradiol on the number of neurons and the extension of neuronal processes (Fig. 1). Incubation of the cultures with the sense oligonucleotide did not significantly affect these parameters [12].

These results indicate that IGF-I synthesis in the cultures is necessary for manifestation of the sex steroid effect, suggesting that estradiol may induce neuronal survival and differentiation by the activation of IGF-I signaling cascades. Several mechanisms may be envisaged to explain the interaction of estradiol and IGF-I. For instance, it has been shown that estradiol may increase transcription of the IGF-I gene by enhancing Fos-Jun binding to the AP-1 motif [64]. However, this hormone does not appear to significantly affect IGF-I synthesis in hypothalamic cultures [56]. Alternatively, estrogen may regulate IGF-I receptors or IGFBPs [56]. In addition, the interaction of estradiol and IGF-I is complicated by the fact that trophic effects of IGF-I on hypothalamic neurons may also be dependent on estrogen receptor.

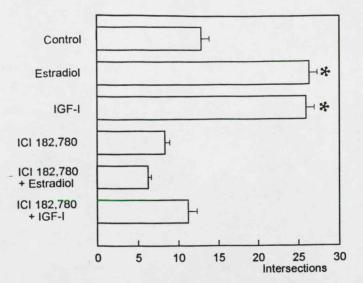


FIG. 2. Effect of the pure estrogen receptor antagonist ICI 182,780 on the growth of MAP-2 immunoreactive processes from hypothalamic neurons in monolayer culture. The presence of ICI 182,780 (10 nM) in the culture medium resulted in the inhibition of the effects of estradiol ( $10^{-9}$  M) and IGF-I ( $10^{-9}$  M). Asterisks indicate significant differences (p < 0.01) vs. cultures incubated in control medium.

### IGF-I MAY ACTIVATE ESTROGEN RECEPTORS IN HYPOTHALAMIC NEURONS

Recent studies indicate that IGF-I [2,26,30,41,51,54] and other trophic factors may activate the estrogen receptor in different cell types. Activation of the estrogen receptor appears to be essential for the mitogenic effect of IGF-I on the prolactin secreting pituitary tumor cell line GH3 [51] and for the IGF-I-induced growth and differentiation of the human neuroblastoma cell line SK-ER3 [41]. In uterine cell cultures transfected with a simple estrogenresponsive reporter gene, not only estrogen, but also IGF-I is able to stimulate estrogen receptor-mediated transactivation and estrogen receptor phosphorylation, suggesting that IGF-I could be acting to stimulate estrogen receptor-mediated transcription in these cells through direct modification of the estrogen receptor protein [2]. Activation of the estrogen receptor occurs in the absence of hormone [2,26,30,41,51,54], is mediated by the membrane-associated receptor tyrosine kinase-Ras-Raf-mitogen-activated protein kinase cascade [30,54], and involves the activation function 2 domain of the estrogen receptor in neuroblastoma cells [54], or the activation function 1 domain in other cell types [26,30].

Based on these studies, we decided to determine if the effect of IGF-I on the survival and differentiation of hypothalamic neurons was dependent on the estrogen receptor. Hypothalamic cultures were grown for 4 days and then incubated with IGF-I (10-48 h in the presence of the pure estrogen receptor antagonist ICI 182,780 (Fig. 2) or an antisense oligodeoxynucleotide to the estrogen receptor [12]. The estrogen receptor antagonist ICI 182,780 blocked the effects of both estradiol and IGF-I on neuronal survival and neuritic growth (Fig. 2). Similar effects were observed when using an antisense oligonucleotide to the estrogen receptor [12]. These parameters were not significantly affected, however, by a nonsense control sequence [12]. These results indicate that estrogen receptors are necessary for the action of IGF-I on hypothalamic survival and neuritic growth [12]. A possible explanation of these results is that IGF-I may activate, either directly or indirectly, estrogen receptors in hypothalamic neurons.

### ESTROGEN ACTIVATION OF HYPOTHALAMIC ASTROGLIA DEPENDS ON IGF-I RECEPTORS

One of the most prominent effects of estradiol on the arcuate nucleus of adult rats is the increase in GFAP levels that occurs in the afternoon of proestrus [20,22,34]. The estrogen-induced increase in GFAP protein and mRNA levels during the afternoon of proestrus is associated with the redistribution of astroglia cytoskeletal components, the growth of astrocyte processes, the ensheathing of neuronal somas by glial processes, and the transient disconnection of inhibitory GABAergic synapses from neuronal somas by the interposed glial processes [22]. These changes are also elicited by the administration of estradiol to adult ovariectomized rats [22].

We have recently tested whether astrocyte activation by estrogen in the arcuate nucleus is dependent on IGF-I (Garcia-Segura et al., unpublished). Hypothalamic tissue fragments from ovariectomized rats, which contained the arcuate nucleus and the median eminence, were incubated in an artificial cerebrospinal fluid for 6 h in the presence or absence of 10<sup>-12</sup> M estradiol. Surprisingly, the hormone did not induce a significant increase in GFAP immunoreactive levels, which is in contrast to what is observed in vivo (Fig. 3). The effect of the hormone was observed, however, in the presence of insulin (10<sup>-6</sup> M), although insulin alone had no effect on GFAP immunoreactivity (Fig. 3). Furthermore, the effect of estradiol in the presence of insulin was abolished when the fragments were incubated with the specific IGF-I receptor antagonist peptide JB1 [55] (1 µg/ml) (Fig. 3). This suggests that the effect of estradiol on arcuate nucleus astrocytes, in the presence of insulin, depends on the activation of IGI-I receptors. Because insulin, at concentrations that act on IGF-I receptors, was unable to induce an increase in GFAP immunoreactive levels, the activation of IGF-I receptors by itself is not enough to stimulate glial cells. This is further support for the existence of a coordinated crosstalk mechanism between the IGF-I and estradiol signaling pathways in the hypothalamus.

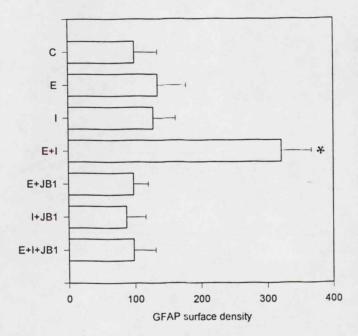


FIG. 3. Effect of estradiol (E), insulin (I) and the IGF-1 receptor antagonist JB1 on GFAP immunoreactive levels in the arcuate nucleus of ovariectomized rats in vitro. The asterisk indicates a significant difference vs. control values (p < 0.01).

### GONADAL STEROIDS REGULATE IGF-I LEVELS IN HYPOTHALAMIC ASTROGLIA

Studies in vivo also suggest an interaction between IGF-I and gonadal steroids and that glial cells actively participate in this interaction. Tanycytes, a specific glial cell type present in the arcuate nucleus and median eminence, are IGF-I immunoreactive [11]. IGF-1 immunoreactive levels in tanycytes show sex differences in the rat arcuate nucleus, with adult females showing significantly lower IGF-I levels than males of the same age. This sex difference is abolished by early postnatal androgenization of females [11], suggesting that it may be dependent on the burst of androgen production by the testis of developing male rats that occurs perinatally. Furthermore, IGF-I levels in tanycytes increase in both male and female rats at the time of puberty. Females show an abrupt increase in IGF-I immunoreactive levels in tanycytes between the morning and the afternoon of the first proestrus. Henceforth, IGF-I immunoreactivity fluctuates according to the different stages of the estrus cycle. IGF-I immunoreactive levels are high in the afternoon of proestrus, after the peak of estrogen in plasma, remain increased in the morning of the following day, and then decrease to basal conditions by the morning of metestrus [11]. In addition, IGF-I levels decrease in tanycytes when gonadal steroid levels are reduced by ovariectomy and increase in a dosedependent manner when ovariectomized rats are injected with 17B-estradiol [11]. The changes in IGF-I levels in tanycytes may be related to the endocrine effects of IGF-I, in particular, with its effects on LHRH secretion [6,25].

### TANYCYTES ARE INVOLVED IN IGF-I UPTAKE FROM BLOOD AND CEREBROSPINAL FLUID

Tanycytes are specialized ependymal cells that are considered a phylogenetically primitive form of astroglia [59]. These cells have many ultrastructural and immunological similarities with astrocytes while preserving a radial shape characteristic of the astroglia of submammalian vertebrates [15,59]. It has been proposed that tanycytes of the arcuate nucleus and median eminence may be involved in endocrine regulation [15,19,40,44]. This function may be mediated in part by tanycyte accumulation of substances from the cerebrospinal fluid or from the capillaries of the median eminence. In agreement with this possibility, previous studies have shown that tanycytes are able to take up  $\beta$ -endorphin from the cerebrospinal fluid [4]. Furthermore, tanycyte processes are closely associated with neurosecretory terminals in the median eminence [35,52]. Encapsulation of neurosecretory terminals by tanycyte endfeet may regulate neuronal contact with portal capillaries [32,33,35,45] and may also result in a focal concentration of factors, such as IGF-I, that may affect neurohormone release. In addition to IGF-I, other factors may be involved in the endocrine effects of tanycytes. For instance, Ma et al. [40] have reported a marked increase in transforming growth factor (TGF)- $\alpha$  gene expression in tanycytes during the initiation of puberty in the rat. TGF-α mRNA levels increase gradually after the anestrous phase of puberty, reaching peak values on the afternoon of the first proestrus. Thus,  $TGF-\alpha$  gene expression in tanycytes fluctuates in parallel to the modifications of IGF-I levels, suggesting a possible interdependence of these changes.

An interesting question in regard to the hormonal-induced changes in IGF-I immunoreactivity in tanycytes is the source of this IGF-I, because tanycytes do not express mRNA for IGF-I [11]. The modifications in IGF-I levels may be the result of the hormonal modulation of its uptake from blood or cerebrospinal fluid. To test for this possibility, IGF-I was labeled with digoxigenin and injected intravenously or in the lateral cerebral ventricle. In both cases we found that various subsets of neurons and glial cells

throughout the CNS, including tanycytes, specifically accumulate the labeled IGF-I (Fernandez-Galaz et al., unpublished). The accumulation of IGF-I was specific because it was substantially decreased by the administration of unlabeled IGF-I or unlabeled insulin, which also acts on IGF-I receptors [38], and was blocked by the specific IGF-I receptor antagonist JB1. The distribution of IGF-I accumulating cells varied, depending on the time after the intravenous administration of labeled IGF-I. By 5 min, IGF-I accumulation was observed in the choroid plexus, median eminence, and area postrema, structures with high local levels of IGF-I receptors [29,39,42,65,66]. By 90 min, IGF-I was accumulated by cells located in different CNS areas such as the olfactory bulb, cerebral cortex, striatum, islands of Calleja, hippocampal formation, habenula, hypothalamus, midbrain, cerebellum, pons, medulla oblongata, and spinal cord. The time elapsed between the intravenous injection of the peptide and the labeling of many brain areas suggest that the peptide is first taken up by areas devoid of blood-brain barrier and then released to the cerebral ventricles. IGF-I may then be accumulated from cerebrospinal fluid by specialized cells such as tanycytes.

### THE ACCUMULATION OF IGF-I BY TANYCYTES FLUCTUATES DURING THE ESTROUS CYCLE

In 1974, Brawer et al. [7] reported ultrastructural modifications in the ventricular surface of tanycytes in the arcuate nucleus of female rats at different stages of the estrous cycle. The number of

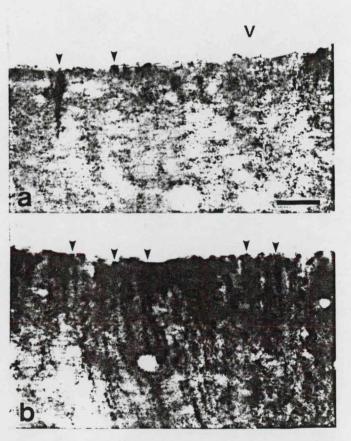


FIG. 4. Labeling of tanycytes (arrowheads) in the arcuate nucleus of female rats after the injection of digoxigenin-IGF-1 in the lateral cerebral ventricle. (A) Afternoon of proestrus (B). Afternoon of estrus, v—Third ventricle. Scale bar, 25  $\mu$ m.

microvilli increased during proestrus remained elevated during estrus and decreased in metestrus. The significance of these changes is still unknown, but may well be related to the uptake of substances from the cerebrospinal fluid. By using colloidal gold ultrastructural immunolocalization techniques, we have recently observed that IGF-I receptors are enriched in the microvilli of tanycytes (Garcia-Segura et al., unpublished). Therefore, changes in the extension of microvilli may be related to changes in the number of IGF-I receptors exposed to the lumen of the third ventricle, and this, in turn, may influence the uptake of IGF-I by tanycytes. To test whether IGF-I uptake by tanycytes fluctuates during the different stages of the estrous cycle, we injected IGF-I labeled with digoxigenin in the lateral cerebral ventricle of cycling female rats. Six animals were injected for each day of the estrous cycle, three of them in the morning and the other three in the afternoon. Animals were killed 1 h after the injection and the number of digoxigenin labeled tanycytes was counted [14].

The number of tanycytes labeled with digoxigenin showed prominent changes associated with the different phases of the estrous cycle (Figs. 4 and 5). Compared to diestrus and metestrus, there was a significant decrease in the number of labeled tanycytes in the afternoon of proestrus. A recovery was observed by the morning of estrus that was followed by a significant increase by the afternoon of estrus. The decrease in the number of tanycytes that accumulate digoxigenin-labeled IGF-I in the afternoon of proestrus was unexpected, because at this stage of the estrous cycle there is a significant increase in IGF-I immunoreactivity in these cells [11]. The apparent discrepancy may be due to the competition of exogenous IGF-I with increased endogenous IGF-I levels in the afternoon of proestrus. Further studies are necessary to determine the cause of the differences observed in the number of tanycytes that accumulate IGF-I after its intracerebroventricular injection. Nevertheless, these findings suggest that tanycytes may regulate the availability of IGF-I to hypothalamic cells.

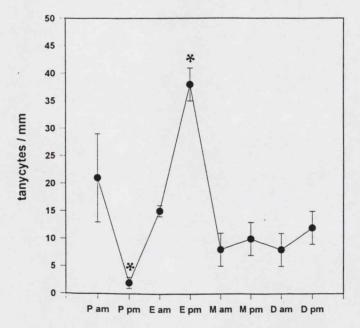


FIG. 5. Number of tanycytes labeled with digoxigenin-IGF-1 during the different stages of the estrous cycle. Data are the number of stained cells per mm of the ependymal cell layer at the level of the arcuate nucleus in the morning (am) and afternoon (pm) of proestrus (P), estrus (E), metestrus (M), and diestrus (D). Asterisks indicate significant differences (p < 0.001) vs. the other stages of the estrous cycle.

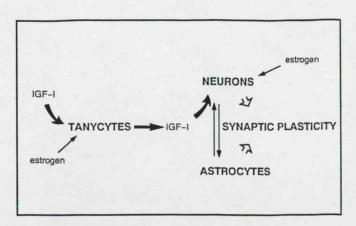


FIG. 6. Diagram depicting the effects of estrogen and IGF-1 on synaptic plasticity in the arcuate nucleus and the interactions between tanycytes, neurons, and astroglia. According to this model, estrogen may affect IGF-1 availability to arcuate neurons by regulating the accumulation of IGF-1 by tanycytes. Estrogen and IGF-1, acting probably on neurons, may regulate neuron to astroglia interactions involved in synaptic plasticity.

#### CONCLUSIONS

The data examined in this review indicate that growth factors. such as IGF-I, participate in the effects of sex hormones in the central nervous system. Gonadal steroids regulate the levels of IGF-I in the mediobasal hypothalamus and the signaling mechanisms of estradiol and IGF-I interact to induce trophic and developmental actions on hypothalamic neurons. Moreover, IGF-I is also involved in activational effects of estradiol on astrocytes of the hypothalamic arcuate nucleus of adult rats. Because activation of astrocytes by estradiol in the arcuate nucleus is linked to modifications in synaptic connectivity, these findings suggest that IGF-I may be involved in estrogen effects on synaptic plasticity. This is further supported by the fact that the modifications in IGF-I levels in the arcuate nucleus of adult females during the estrous cycle correlate with plastic changes in synaptic connectivity. Moreover, IGF-I in the arcuate nucleus of adult rats appears to be originated, at least in part, in extrahypothalamic areas. Tanycytes. a specialized form of hypothalamic astroglia, may play a crucial role in the local effects of this factor by regulating its availability to hypothalamic cells. In summary, IGF-I and different forms of glia to neuron communication appear to be involved in the effects of gonadal hormones on the neuroendocrine hypothalamus. A diagram depicting the interactions between these various components is shown in Fig. 6. According to this model, estrogen will regulate IGF-I levels in the arcuate nucleus by acting on tanycytes. IGF-I may then affect estrogen-induced neuron to astrocyte signaling mechanisms involved in synaptic plasticity.

#### **ACKNOWLEDGEMENTS**

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# Nitric oxide modulates the gastrointestinal plasma extravasation following intraabdominal surgical manipulation in rats

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#### Abstract

The actions of nitric oxide (NO) on gastrointestinal plasma loss, assessed by the leakage of [ $^{123}$ I]human serum albumin, provoked by intraabdominal surgery and organ manipulation has been investigated in pentobarbitone-anaesthesized rats. Gentle manipulation (3 min) of the stomach or the small intestine following laparotomy leads to an increase in albumin extravasation in the stomach, duodenum, jejunum and colon over 1 h. Administration of the NO synthase inhibitors,  $N^G$ -nitro-L-arginine methyl ester (1–5 mg kg $^{-1}$ , s.c.) and  $N^G$ -monomethyl-L-arginine (12.5–50 mg kg $^{-1}$ , s.c.), provoked a further substantial elevation of gastrointestinal albumin extravasation in the surgically manipulated rat. but not in control rats. This effect could be prevented by the pretreatment (15 min) with L-arginine (300 mg kg $^{-1}$ , s.c.) or by the concurrent infusion of the NO donor, S-nitroso-glutathione (5  $\mu$ g kg $^{-1}$  min $^{-1}$ , i.v.). Endogenous NO, most likely formed by endothelial NO synthase, thus appears to maintain microvascular integrity during surgery and organ manipulation of the gastrointestinal tract. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Nitric oxide (NO) endothelial; Vascular permeability; Gastrointestinal tract; Surgery; Plasma loss; Microclyculation

#### 1. Introduction

Experimental and clinical studies have shown that a reduction in circulating plasma volume can occur during or following major surgical intervention (Jarnum, 1961; Krakelund, 1971; Robarts, 1979; Akerström and Lisander, 1991). However, there is no clear understanding of the mechanism of such plasma extravasation associated with surgical manipulation or the processes that regulate it 'Akerström and Lisander, 1991).

Nitric oxide (NO), formed continuously in the vascular endothelium and in neuronal elements by the constitutive NO synthase (endothelial NO synthase and neuronal NO synthase, respectively), plays a key role in the maintenance of microvascular integrity under physiological circumtances (Moncada et al., 1991; Moncada and Higgs, 1995). However, under pathological conditions (such as following andotoxin or cytokine administration), the widespread exression of the inducible NO synthase can be detected in

The expression of inducible NO synthase provokes microvascular leakage of albumin into gastrointestinal tissues following endotoxin administration (Boughton-Smith et al., 1993). Moreover, the selective inhibition of inducible NO synthase by the bisisothiourea derivative, 1400 W, protects the microvasculature against such plasma loss (László and Whittle, 1997). Thus, the uncontrolled production of NO under pathological conditions is detrimental towards the vascular endothelium, which may involve the generation of such tissue damaging radicals as the hydroxyl and peroxynitrite (Beckman et al., 1990; Hogg et al., 1992; Lipton et al., 1993).

In contrast, the physiological generation of NO by endothelial NO synthase has beneficial microcirculatory effects. Thus, inhibition of endothelial NO synthase by the administration of the nonselective NO synthase inhibitor,  $N^G$ -nitro-L-arginine methyl ester (L-NAME) or  $N^G$ -monomethyl-L-arginine (L-NMMA), provokes intestinal albumin leakage following challenge with low doses of endotoxin,

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many cell types including the vascular endothelium, neutrophils, macrophages and intestinal epithelial cells which leads to the overproduction of NO (Moncada et al., 1991; Moncada and Higgs, 1995).

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under conditions where neither endotoxin nor NO synthase inhibitors alone affected microvascular albumin extravasation (László et al., 1994a). Furthermore, L-NMMA augmented gastrointestinal microvascular damage induced by high doses of endotoxin (Hutcheson et al., 1990). Constitutive NO synthase has a beneficial role during the initiation of intestinal inflammation, since its inhibition in the early phase of various inflammatory bowel disease models provokes or augments microvascular leakage (Kiss et al., 1997; László and Whittle, 1998).

Endogenous NO attenuates the adherence and immigration of leukocytes in the vascular endothelium (Kubes et al., 1991), actions that have key importance in the generation of vascular permeability and inflammation (Wedmore and Williams, 1981). Indeed, administration of inhibitors or antagonists of such neutrophil-derived mediators as platelet-activating factor, thromboxanes and leukotrines attenuated intestinal microvascular injury provoked by the inhibition of endothelial NO synthase in the early phase of experimental sepsis and intestinal inflammation (László et al., 1994b; László and Whittle, 1998).

In a recent study, following the administration of the NO synthase inhibitors. L-NAME or L-NMMA, abdominal laparotomy produced a significant elevation in microvascular leakage in the jejunum and colon over 1 h (László and Whittle, 1999). These NO synthase inhibitors had no such effect in conscious, anasthesized or skin-incised rats. In addition, no significant difference in albumin leakage and accumulation was observed in tissues from unoperated conscious or anaesthesized rats, or in anaesthesized rats with a skin-incision or abdominal laparotomy under resting conditions, showing that these minor surgical interventions alone do not provoke changes in microvascular permeability to albumin.

In many studies on the microcirculation, the tissues under investigation are exposed by laparotomy. Moreover, in such studies, the organs such as those of the gut, are further manipulated or exteriorised, to expose a suitable region for the observation. Thus, in the present study, we have now extended the findings with laparotomy alone, to evaluate the effects of abdominal surgery with gentle manipulation of the gastrointestinal organs on vascular permeability, and to study the effects of inhibitors of NO synthase.

#### 2. Materials and methods

#### 2.1. Surgical manipulation

Male Wistar rats (225-275 g) were fasted overnight, but allowed for free access to water. The animals were separated into two groups:

In the control group, the animals were deemed to be conscious for the majority of the experimentation period, since the treatments were performed under transient halothane anaesthesia from which the animals had completely recovered within 2 min. Autopsy in this group verformed under halothane ungesthesia within 1 min.

In the laparotomy and organ manipulated groups (tern surgical manipulation), the animals received pentobal tone (60 mg kg<sup>-1</sup>; i.p.) to induce annesthesia and we tracheotomized. A 5-cm-long midline laparotomy in 1 abdominal wall was performed, without significant blecking. In the manipulated group, part of the small bowel we exteriorised and gently handled for 3 min in a 5 × 5 cm gauze pad moistened in 37°C saline. The bowel was placed back into the abdominal cavity, and a gauze moistene with saline was placed over the incision for the protection against evaporation. Those talls who had significant bleeding were excluded from the study. In all of the anaes thetised groups, the body temperature was maintained of 36.5–37°C using a homeothermic control unit and under blanket (Harvard Instruments).

[125] I human serum albumin (2 μCl kg<sup>-1</sup>, i.v.) was administered via a needle inserted into the tail vein, and autopsy was performed 1 h later, i.e., the time-interval between the start of surgical procedures and autopsy was 60 min. The 1 h maximum timepoint was chosen to exclude the involvement of inducible NO synthase, that requires 2–3 h following challenge for expression, since this could modify vascular leakage and hence confound interpretation of the findings (Boughton-Smith et al., 1993).

#### 2.2. Plasma leakage

As a measure of vascular endothelial permeability, leakage of [125] Ilhuman serum albumin into tissue was determined in segments of the stomach, duodenum, jejunum and colon. Blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0.318%) and centrifuged (10,000  $\times$  g, 10 min. 4°C). The [125] human serum albumin content of the plasma and segments of tissues was determined in a gamma-spectrometer (Nuclear Enterprises NE 1600) and the albumin content in tissues was calculated. The control value for albumin accumulation was taken as the mean of the data of a group of control unanaesthesized animals, which received albumin only, which reflects basal albumin movement into tissues. This basal control mean value was calculated and subtracted from the value from each of the animals in each experimental group. The data were expressed as changes in albumin accumulation (A plasma leakage, µl plasma g-1 tissue), corrected for intravascular volume as described previously (Boughton-Smith et al., 1993; László et al., 1994a).

# 2.3. Effect of L-NAME and L-NMMA on gastrointestinal plasma leakage

In a set of rats from each of the groups, L-NAME (1-5 mg kg<sup>-1</sup>, s.c.) or L-NMMA (1-50 mg kg<sup>-1</sup>, s.c.) was injected concurrently with [125 I]human serum albumin. Plasma leakage in the jejunum and colon was evaluated

after 1 h. In a separate group, rats were pretreated with L-arginine (300 mg kg<sup>-1</sup>, s.c.) 15 min before L-NAME (5 mg kg<sup>-1</sup>, s.c.) administration, and gastric, duodenal, jejunal and colonic plasma leakage was determined 1 h after L-NAME.

# 2.4. Effect of S-nitroso-glutathione on gastrointestinal plasma leakage

In surgically manipulated rats, infusion of the NO donor S-nitroso-glutathione (5 µg kg<sup>-1</sup> min<sup>-1</sup>) into the tail vein was commenced concurrently with the administration of L-NAME (5 mg kg<sup>-1</sup>, s.c.). Plasma leakage in the stomach. duodenum, jejunum and colon was measured 1 h later.

#### 2.5. Chemicals

[125 I]human serum albumin was obtained from Amersham International (UK) and IZINTA (Budapest, Hungary). All the other compounds were from Sigma (Poole, Dorset, UK).

#### 2.6. Statistics

The data are expressed as mean  $\pm$  S.E.M. from (n) rats per experimental group. For statistical comparisons, analysis of variance with the Bonferroni test was utilised, where P < 0.05 was taken as significant.

#### 3. Results

# 3.1. Effect of surgical manipulation on gastrointestinal plasma leakage

Abdominal surgery and gentle manipulation of the organs provoked significant albumin leakage into the gastric and duodenal tissue (Fig. 1), and jejunal and colonic

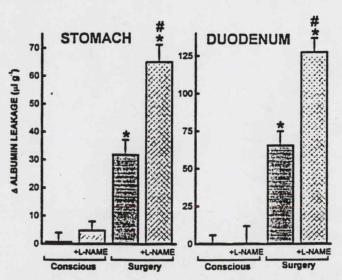


Fig. 1. Leakage of radiolabelled albumin (expressed as  $\Delta$  albumin leakage,  $\mu l g^{-1}$  tissue) provoked by intraabdominal surgery and manipulation in the stomach and duodenum of the rat, and its aggravation by  $N^{\rm C}$ -nitro-L-arginine methyl ester (L-NAME, 5 mg kg<sup>-1</sup>, s.c.). Data are shown as the mean  $\pm$  S.E.M., where (n) is 8-12 for each group, and where statistical significance is given as \*P < 0.05 compared to conscious untreated groups.

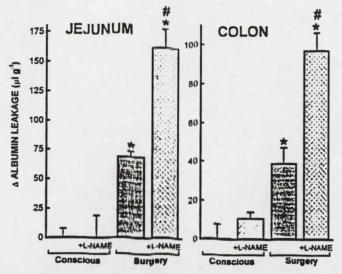


Fig. 2. Leakage of radiolabelled albumin (expressed as  $\Delta$  albumin leakage,  $\mu l g^{-1}$  tissue) provoked by intraabdominal surgery and manipulation in the jejunum and colon of the rat, and its aggravation by  $N^G$ -nitro-L-arginine methyl ester (L-NAME, 5 mg kg<sup>-1</sup>, s.c.). Data are shown as the mean  $\pm$  S.E.M., where (n) is 8-12 for each group, and where statistical significance is given as \*P < 0.05 compared to conscious untreated groups.

tissues (Fig. 2) over 1 h. compared with the control (resting) albumin extravasation in the stomach, duodenum, jejunum and colon of the conscious rat. The control values of plasma leakage are  $51 \pm 3$ ,  $118 \pm 5$ ,  $136 \pm 8$  and  $52 \pm 7$   $\mu l g^{-1}$  tissue, respectively, over this 1 h period.

## 3.2. Effect of L-NAME or L-NMMA on gastrointestinal plasma leakage following surgical manipulation

In conscious unoperated control rats, administration of the NO synthase inhibitor, L-NAME (5 mg kg<sup>-1</sup>, s.c.) did

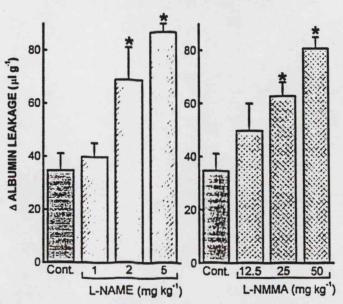


Fig. 3. Leakage of radiolabelled albumin (expressed as  $\Delta$  albumin leakage,  $\mu$ l g<sup>-1</sup> tissue) provoked by intraabdominal surgery and manipulation in the colon of the rat, and its dose-dependent aggravation by  $N^G$ -nitro-L-arginine methyl ester (L-NAME, 1-5 mg kg<sup>-1</sup>, s.c.) and by  $N^G$ -monomethyl-L-arginine (L-NMMA, 12.5-50 mg kg<sup>-1</sup>, s.c.). Data are  $\tau$  shown as the mean  $\pm$  S.E.M., where (n) is 6-12 for each group, and where statistical significance is given as  $^*P < 0.05$  compared to the surgically manipulated control (Cont.) groups.

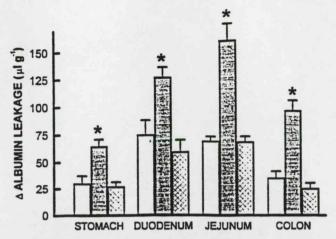


Fig. 4. Leakage of radiolabelled albumin (expressed as  $\Delta$  albumin leakage,  $\mu$ l  $g^{-1}$  tissue) provoked by intraabdominal surgery and manipulation in the stomach, duodenum, jejunum and colon of the rat (opened columns), and its aggravation by  $N^G$ -nitro-L-arginine methyl ester (L-NAME, 5 mg kg<sup>-1</sup>, s.c., grey columns). Inhibition of albumin leakage provoked by L-NAME in the surgically manipulated rat (hatched columns) by S-nitroso-glutathione infusion (5  $\mu$ g kg<sup>-1</sup> min<sup>-1</sup>, i.v.). Data are shown as the mean  $\pm$  S.E.M., where (n) is 6–9 for each group, and where statistical significance is given as \*P < 0.05 compared to the intraabdominal surgery alone (open columns) groups.

not affect plasma leakage over 1 h in the gastric and duodenal tissue (Fig. 1), or in jejunal and colonic tissues . (Fig. 2).

In surgically manipulated rats, administration of NO synthase inhibitors, L-NAME (1-5 mg kg<sup>-1</sup>, s.c.) or L-NMMA (12.5-50 mg kg<sup>-1</sup>, s.c.) provoked a further dose-dependent increase in plasma leakage over 1 h in the stomach and duodenum (Fig. 1), in the jejunum (Fig. 2) and in the colon (Figs. 2 and 3). This augmentation of gastrointestinal plasma leakage was inhibited near-maximally with L-arginine (300 mg kg<sup>-1</sup>, s.c.) pretreatment (15 min before L-NAME) by  $100 \pm 12\%$ ,  $90 \pm 10\%$ ,  $91 \pm 21\%$  and  $92 \pm 19\%$  in the stomach, duodenum, jejunum and colon, respectively (n = 4-6, P < 0.005).

# 3.3. Effect of S-nitroso-glutathione on gastrointestinal plasma leakage

Intravenous infusion of the NO donor, S-nitrosoglutathione (5 µg kg<sup>-1</sup> min<sup>-1</sup> for 1 h) abolished the increase in gastric, duodenal, jejunal and colonic plasma leakage induced by L-NAME (5 mg kg<sup>-1</sup>, s.c.) in surgically manipulated rats, as shown in Fig. 4.

#### 4. Discussion

In this present study, an increase in vascular permeability has been observed in gastric, duodenal, jejunal and colonic tissues in rats that had undergone intraabdominal surgery and gentle manipulation of the gastrointestinal tissues. Our study confirms previous findings in which it

has shown that abdominal surgery itself provoked an vation of vascular permeability in rats (Akerström Lisander, 1991). The increase in albumin extravasa under the current conditions is a consequence of the general procedures of tracheotomy, incision of the abdominal skin or abdominal laparotomy alone did not provoalbumin leakage in the anaesthetised rat (László and Whole, 1999). Thus, it is feasible that our model is appropriate the investigation of the mechanisms of plasma leaduring surgical procedures.

Administration of the NO synthese inhibitor, L-NAN in conscious unoperated rate did not affect basal album extravasation within the 1 h period, which is consiste with our previous findings (László et al., 1994a,b). How ever, when the NO synthase inhibitors were administer. in rats with surgery and tissue manipulation, a furthincrease in gastrointestinal vascular permeability occurre The actions of L-NAME could be reversed by L-arginia pretreatment. It is likely that actions of endothelial No synthase involved, since previous studies have demon strated that a minimum of 2 h are needed for the expres sion of the inducible NO synthase (Boughton-Smith et al. 1993), while in our current model, the experimental period has been only 1 h. On the basis of our present data, in appears that NO produced by endothelial NO synthas modulates the changes in vascular permeability durin surgical challenge. Such events include abdominal laparo tomy itself (László and Whittle, 1999) and subsequen organ manipulation. Thus, NO generated by endothelia. NO synthase may play a protective role in the microcirculation against endothelial dysfunction and the consequent plasma loss during and following surgical operations.

Physiologically formed NO might protect the microcirculation by preventing the deleterious actions of neutrophils towards the vascular endothelium during surgical procedures. Polymorphonuclear leukocytes are well-known to play a crucial role in the changes in microvascular permeability during inflammatory processes leading to tissue oedema (Wedmore and Williams, 1981). Administration of L-NAME enhances the adhesion of leukocytes to the vascular endothelium, assessed by in vivo microscopy in surgically prepared animals (Kubes et al., 1991; Arndt et al., 1993). In a recent study, the increase in plasma leakage by L-NAME, in rats with abdominal laparotomy alone, was abolished by the pretreatment of a rabbit anti-rat neutrophil serum suggesting the involvement of neutrophils in these events (László and Whittle, 1999). Thus. endogenous NO formed by endothelial NO synthase may counteract to the effects of neutrophil-derived mediators which are released in response to surgical trauma.

In our present study, administration of the NO donor, S-nitroso-glutathione attenuated plasma extravasation provoked by L-NAME and surgical manipulation. This protection by the NO donor may involve actions on neutrophils, since NO decreases neutrophil function and their adhesion

to the vascular endothelium both in vivo and in vitro (Ma et al., 1993; Moilanen et al., 1993; Granger and Kubes, 1994).

Our present results thus suggest that the minor surgical intervention of opening the abdominal cavity and a gentle handling of the stomach or the small intestine lead to microvascular changes in these tissues. This process appears to be modulated by NO, since inhibition NO synthase substantially increased plasma extravasation from the gastrointestinal organs under these conditions. The mechanisms underlying this apparent microvascular priming are unknown, but may reflect neuronal or humoral stimulation as a consequence of the surgical stress, as well as the activation of the neutrophil. The current data suggest that should NO formation be compromised during surgical procedures, plasma and fluid loss would be augmented, events which could lead to hypovolaemia, as well as oedema formation and a decreased tissue oxygenisation. It is possible therefore that administration of NO donors during major surgical interventions may be beneficial in preventing any subsequent vascular endothelial dysfunction and the consequent plasma loss.

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Szepes Z., <u>Morschl É</u>., 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.

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# Detrimental effects of oestradiol on cysteamine-induced gastroduodenal ulceration in the female rat

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Abstract — The actions of the female sex steroid, oestradiol on cysteamine-induced mucosal ulceration has been evaluated in female Wistar rats. Administration of cysteamine (400 mg·kg<sup>-1</sup>, s.c.) provoked macroscopic gastroduodenal mucosal injury (assessed planimetrically) and an increase in microvascular permeability (assessed by the extravasation of radiolabeled alloumin) in the stomach and duodenum, determined 24 h later. Ovariectomy (2 weeks before cysteamine) reduced gastroduodenal macroscopic injury, and albumin extravasation following cysteamine challenge. Administration of oestradiol (1–5 mg·kg<sup>-1</sup>, as an i.m. depot 1 week before cysteamine) dose-dependently augmented gastric and duodenal macroscopic mucosal lesions and microvascular hermeability provoked by cysteamine. These findings indicate that oestradiol can exacerbate gastroduodenal ulceration and microvascular injury. © 1999 Elsevier Science Ltd. Published by Éditions scientifiques et médicales Elsevier SAS

sex steroids / ovariectomy / oestradiol / mucosal ulceration / micro-vascular permeability / cysteamine

#### 1. Introduction

On the basis of experimental and clinical observations, it is known that ulceration of the gastroduodenal mucosa shows sexual dimorphism. Thus, in the fertile age, peptic ulcer disease occurs more frequently among men than women [2]. In experimental models of mucosal damage, this sexual difference of mucosal injury has also been found. Oral administration of ethanol caused more severe gastric mucosal erosions in male rats than in females [8, 9]. In this model of mucosal damage, gonadectomy protected the stomach against ethanol-induced injury in male rats [8]. Moreover, administration of the testosterone-synthase inhibitor, cyproterone acetate attenuated gastric hemorrhagic erosions in intact male rats following ethanol challenge [8], and reduced gastric lesions and duodenal ulceration provoked by cysteamine [11]. Such findings suggest that endogenous sex hormones might possibly play a role in the development of gastroduodenal mucosal ulceration.

The aim of the present study was to evaluate the actions of the female sex steroid, oestradiol on the generation cysteamine-induced gastroduodenal ulceration in the rat. Thus, in female rats, we examined the effects of ovariectomy and oestradiol on the severity of macroscopically detectable lesions as well as the changes in microvascular permeability in the stomach and duodenum provoked by cysteamine.

#### 2. Materials and methods

#### 2.1. Experimental protocol

Female Wistar rats (200–220 g) were injected with cysteamine (400 mg·kg<sup>-1</sup>, s.c.). Twenty-four hours after cysteamine administration, the animals were killed by ether overdose, and their stomach and duodenum were removed. During this 24-h period, rats were deprived of food, but received water ad libitum. For the determination of plasma leakage, <sup>125</sup>I-labeled human serum albumin ([<sup>125</sup>I]-H\$A, 2 µCi·kg<sup>-1</sup>, i.v.) was administered 2 h before autopsy. In some groups of rats, ovariectomy was performed under ether anaesthesia 2 weeks before any experimental procedure. For additional groups of rats, a depot injection of oestradiol (1–5 mg·kg<sup>-1</sup>, i.m.) were administered 1 week before cysteamine challenge.

#### 2.2 Macroscopic evaluation of lesions

The area of damage and the total mucosal surface of the stomach and duodenum were measured by a digital planimeter (Sokkia Planimeter Kl'-82-N, Japan), and the injured area was expressed as percentage of the total mucosal surface.

#### 2.3. Albumin leakage

Leakage of [125]]-HSA was determined in the stomach and duodenum as an index of microvascular damage. Blood was collected from the abdominal aorta into syringes containing trisodium citrate (final



<sup>\*</sup> Correspondence and reprints

concentration 0.318%) and centrifuged (10 000 g, 10 min, 4 °C). The [ $^{125}\text{I}$ ]-HSA content of the plasma and segments of gastric and duodenal tissue was determined in a gamma-spectrometer (Nuclear Enterprises NE 1600) and the albumin content in the stomach and duodenum calculated, taking into account any changes in gastroduodenal blood volume as described previously [4]. Values from control tissues were subtracted from the values of treated tissue and the data were expressed as plasma leakage,  $\mu L$  plasma g $^{-1}$  wet tissue.

#### 2.4. 17-β-oestradiol plasma level

Plasma 17- $\beta$ -oetradiol levels were determined after ovariectomy by a radioimmuno-assay method described previously [8].

#### 2.5. Materials

[125]]-labeled human serum albumin was obtained from Izinta (Budapest, Hungary). Oestradiol benzoate was purchased from Intervet International (Boxmeer, the Netherlands). All other compounds were from Sigma Chemical Company.

#### 2.6. Statistical evaluation

The data are expressed as mean  $\pm$  SE from (n) rats per experimental group. For statistical comparisons, Student's *t*-test test for unpaired data or analysis of variance with the Bonferroni test were used, where P < 0.05 was taken as significant difference.

#### 3. Results

## 3.1. Gastroduodenal ulceration provoked by cysteamine

Administration of cysteamine (400 mg·kg<sup>-1</sup>, s.c.) provoked macroscopic injury in the gastric and duodenal mucosa (4.5  $\pm$  0.6% and 12.9  $\pm$  1.0% of total mucosal area, respectively; n = 7, P < 0.001) as shown in figure 1. Cysteamine challenge also increased microvascular permeability in the stomach ( $\Delta 57 \pm 9 \, \mu \text{L} \cdot \text{g}^{-1}$  tissue, n = 7, P < 0.001) and duodenum ( $\Delta 155 \pm 6 \, \mu \text{L} \cdot \text{g}^{-1}$  tissue; n = 7, P < 0.001) after 24 h, as shown in figure 2.

#### 3.2. Effects of ovariectomy

Ovariectomy (2 weeks before cysteamine challenge) decreased plasma 17- $\beta$ -oestradiol level (from  $182 \pm 10$  to  $87 \pm 5$  pmol·L<sup>-1</sup>, n = 6). Ovariectomy significantly lowered the gastric and duodenal macroscopic mucosal injury provoked by cysteamine (by

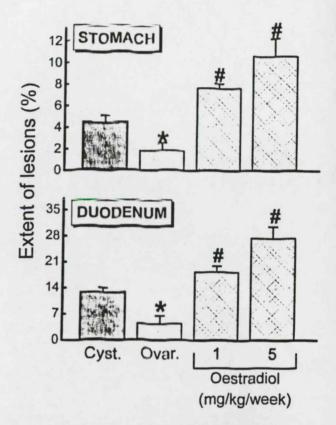


Figure 1. Induction of rat gastric and duodenal mucosal macroscopic injury by cysteamine (Cyst. 400 mg·kg<sup>-1</sup>, s.c.) after 24 h. The inhibition of cysteamine-induced damage by ovariectomy (Ovar., 2 weeks before cysteamine challenge) and the potentiation of cysteamine-provoked mucosal lesions by oestradiol (1–5 mg·kg<sup>-1</sup>·week<sup>-1</sup>, i.m.) pretreatment is shown. The columns show the extent of gastric and duodenal macroscopic lesions (expressed as lesion area, percentage total mucosal area). Data are given as the mean  $\pm$  SE of 4–7 rats per group; statistical significance is shown as \*P < 0.05, inhibition of cysteamine-induced lesion; "P < 0.05, potentiation of cysteamine-induced lesion.

 $62 \pm 13\%$  and  $65 \pm 15\%$ , respectively; P < 0.001, n = 5) as shown in figure 1. Ovariectomy likewise attenuated albumin extravasation in the gastric mucosa and duodenum provoked by cysteamine (by  $63 \pm 19\%$  and  $65 \pm 7\%$ , respectively; P < 0.001, n = 5) as shown in figure 2.

#### 3.3. Effects of oestradiol

Administration of oestradiol (1–5 mg·kg<sup>-1</sup>·week<sup>-1</sup>, i.m.) to unoperated rats dose-dependently augmented gastric and duodenal macroscopic mucosal lesions induced by cysteamine (by  $138 \pm 35\%$  and by  $114 \pm 23\%$ , respectively; P < 0.001, n = 4). This treatment also increased the microvascular injury in the stomach and duodenum (by  $182 \pm 59\%$  and by

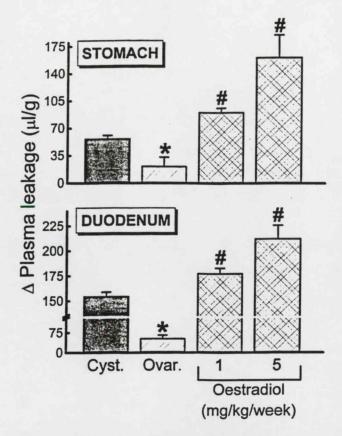


Figure 2. Induction of rat gastric and duodenal vascular leakage of plasma (determined using radiolabeled albumin) by cysteamine (Cyst., 400 mg·kg<sup>-1</sup>, s.c.) after 24 h. The inhibition of cysteamine-induced plasma leakage by ovariectomy (Ovar., 2 weeks before cysteamine challenge) and potentiation of cysteamine-provoked plasma leakage by oestradiol (1–5 mg·kg<sup>-1</sup>·week<sup>-1</sup>, i.m.) pretreatment is shown. The columns show the leakage of plasma ( $\Delta$   $\mu$ L·g<sup>-1</sup> tissue) in gastric and duodenal tissues. Data are given as the mean  $\pm$  SE of 4–7 rats per group; statistical significance is shown as \*P < 0.05, inhibition of cysteamine-induced plasma leakage; \*P < 0.05, potentiation of cysteamine-induced plasma leakage.

 $38 \pm 5\%$ , respectively; P < 0.01, n = 4) provoked by cysteamine (figures 1 and 2, respectively).

#### 4. Discussion

This study in female rats following ovariectomy extends the previous observations in males that following gonadectomy the gastroduodenal mucosa is less sensitive to various ulcerogenic stimuli [8, 11]. The reduction by ovariectomy of cysteamine-induced ulcers of the stomach and duodenum could be related to the observed decrease of plasma oestradiol levels. Indeed, mucosal damage in response to challenge was

increased by exogenous administration of oestradiol into intact female rats.

It is known that an increase of gastric parietal cell mass has a beneficial action against the development of mucosal lesions following ulcerogenic challenge and accelerates ulcer healing [6, 7]. Since ovaricctomy enhances gastric parietal cell mass [1], this process may contribute to the mechanism by which ovariectomy protected the mucosa against cysteamine-induced injury. Other factors are also likely to be involved in the apparent aggressive actions of oestradiol towards the gastroduodenal mucosa. Thus administration of oestrogens can lead to the inactivation of prostaglandins in the stomach [5], and prostaglandins are known to have key importance in the defense mechanism of the gastroduodenal mucosa [13, 16].

Here, cysteamine challenge also provoked microvascular plasma leakage, as a measure of vascular endothelial damage. This finding is in agreement with the previous suggestion that aggressive vascular factors are involved in the generation of cysteaminegastroduodenal ulcers [3]. It is wellinduced established that microcirculatory injury and the consequent hypoxia are among those important factors which are considered to lead to gastroduodenal mucosal ulceration [14, 15]. As with the macroscopic injury, in our present work, the reduction in endogenous oestradiol synthesis following ovaricctomy lead to the attenuation of cysteamine-induced microvascular leakage. In contrast, augmentation of microvascular leakage followed the administration of exogenous oestradiol after cysteamine challenge. Although further studies are needed to evaluate whether the observed microvascular defects are the consequence or the cause of cysteamine-provoked mucosal injury, it is relevant that oestrogens have vasoconstrictor actions by modulating the pressor effect of vasopressin [12]. The significant aggressive role of endogenous vasopressin via its pressor receptors in the development of gastroduodenal mucosal ulceration has been described previously [10, 12].

In conclusion, oestradiol appears to exert a proulcerogenic action in the generation of macroscopic and microvascular injury in the stomach and duodenum following cysteamine administration in female rats. Thus inappropriate levels of endogenous oestradiol might possibly be involved in the pathogenesis of gastroduodenal mucosal damage.

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Estrogen-mediated up-regulation of the Ca-dependent constitutive nitric oxide synthase in the rat aorta and heart.

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Life Sciences

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## Estrogen-mediated up-regulation of the Ca-dependent constitutive nitric oxide synthase in the rat aorta and heart

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#### **Abstract**

The role of endogenous estrogens has been studied in the regulation of the Ca-dependent constitutive nitric oxide synthase (cNOS) enzyme activity in aortic and cardiac tissues of the rat. The activity of cNOS enzyme was measured by the citrulline assay in the abdominal aorta and in the left ventricle of the heart obtained from male, sham-operated female and ovariectomized female Wistar rats. Estrogen replacement therapy (17-β-estradiol, 20–100 μg/kg/day, s.c.) has been performed in ovariectomized rats over two weeks. We found that cNOS activity was higher in the aorta and heart of female rats compared to males. Ovariectomy decreased cNOS activity in both tissues to that level what could be observed in males. Estrogen supplementation 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 the levels found in females. We concluded that endogenous estrogens up-regulate the activity of the cNOS isoenzymes in the rat aorta and heart. © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Nitric oxide; eNOS enzyme regulation; Estrogen; Cardiovascular protection; Ischemic heart disease

#### Introduction

In the reproductive years, the incidence of ischemic heart disease among women is lower compared to men [1]. 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 male [2]. 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 [1]. Moreover, the risk of ischemic heart disease is significantly higher among surgi-

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cally postmenopausal women in comparison with pre-menopausal women in the same age [3]. Population-based observational studies showed that estrogen replacement therapy in postmenopausal women reduced the mortality due to cardiovascular disease [4]. These data suggest the cardiovascular protective role of endogenous and exogenous estrogen.

The cardiac protective effect of estrogen, at least in part, relates to the actions of the hormone on blood lipid profiles. Estrogen decreases serum low-density lipoprotein and increases high-density lipoprotein cholesterol concentration. It is clear that such changes in serum lipoprotein concentrations are important factors in the development of coronary artery and generalized atherosclerosis. Indeed, direct experimental findings showed that estrogen replacement therapy reduced the extent of coronary artery atherosclerosis in ovariectonized monkey [5]. In addition, it was also shown that estrogen replacement therapy increases endothelium-dependent relaxation in the coronary artery under experimental and clinical circumstances [6,7]. On the basis of these observations, it is suspected that the major site of the cardiovascular protective actions of estrogen is the arterial intima where most likely vuscular endothelium-dependent mechanisms are involved.

Nitric oxide (NO) is synthesized from L-arginine by three distinct isoenzymes. These NO synthase (NOS) enzyme isoforms can be divided into two functional classes based on their sensitivity to calcium. The cytokine- or bacterial endotoxin-inducible isoenzyme (iNOS) binds calmodulin tightly in a calcium-independent fashion, and expressed mostly under pathological conditions. The constitutive forms (cNOS), isoenzymes endothelial NOS (eNOS; originally described in vascular endothelial cells) and neuronal NOS (nNOS; originally described in neuronal tissues), bind calmodulin in a reversible and calcium-dependent fashion. NOS enzyme activities in tissues can be determined by the citrulline assay, what we used in our present study, based on the functional difference in their enzymatic parameters between iNOS and cNOS. As the two isoenzymes of the calcium-dependent constitutive NOS (eNOS and nNOS) can not been differentiated, therefore, we use later the traditional collective term cNOS. Under physiological circumstances the activity of cNOS can be detected continuously, and plays a crucial cardiovascular protective role. However, the mechanisms by which the activity of the cNOS isoenzymes is regulated are poorly understood [8,9].

A number of indirect *in vitro* (vascular endothelial cell culture, isolated organs, etc.) observations have suggested that the liberation of NO might be regulated by sex hormones [10]. In our present study, we investigated the actions of endogenous estrogens, directly on the regulation of cNOS enzyme activity under *in vivo* circumstances in the aorta and even in the heart (since no data still available in this organ) obtained from the conscious surgically postmenopausal (i.e. ovariectomized) rat.

#### Methods

Study groups

10-12 weeks-old male and female Wistar rats were used. Ovariectomy and sham operation have been performed under ether anesthesia. The animals were allowed to recover over one month. In separate groups of ovariectomized animals, estrogen replacement therapy (17- $\beta$ -estradiol, 20-100  $\mu$ g/kg/day, s.c.) was introduced over a two-week period.

#### Ca-dependent nitric oxide synthase enzyme activity

NOS activity was determined as the conversion of L-[14C]-arginine monohydrochloride to L-[14C]-citrulline based on the method described previously [11,12] with minor modifications aiming to detect mostly the activity of cNOS [10,13,14]. We sacrificed the unimals by decapitation, and immediately after autopsy, we prepared the fresh tissues for NOS measurements. Cardiac (the left ventricle from an individual rat) and aortic (pooled from the abdominal aorta of two rats) tissues were homogenized (15 s, Ultra-Turrax homogenizer, 5 mm blade) in buffer (250 mg/ml, 4°C, 10 mM Hepes, 32 mM sucrose, 1 mM dithiothrcitol, 0.1 mM EDTA, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml of leupeptin, and 2 µg/ml of aprotonin, pH 7.4), and was followed by centrifugation for 20 min at 10,000 g at 4 °C. Samples were mixed with Dowex (AG 50W-8; 200-400, 8% cross-linked, Na<sup>+</sup> form) resin, and followed by centrifugation for a further 10 min at 10,000 g at 4 °C. Sample supernatunt (4() μl) was incubated for 10 min at 37°C in reaction buffer comprising (final concentrations) 50 mM KH<sub>2</sub>PO<sub>4</sub>, 10 μg/ml calmodulin, 2.5 mM CaCI<sub>2</sub>, 50 mM valine, 1 mM dithiothreitol, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 µM flavin adenine dinucleotide (FAD), 3 μM flavin mononucleotide (FMN), 3 μM tetrahydrobiopterin and 0.17 μM of [14C]Larginine. 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 allowing the 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 us pmol/ min/mg protein, where constant linear rates with different amounts of extracts could be found. Total NOS activity was defined as citrulline formation that was abolished by incubation in vitro with NG-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, calciumindependent NOS activity (iNOS) was also determined as the difference between samples containing 1mM EGTA and samples containing 1 mM L-NNA.

#### Chemicals and statistics

L-[U- $^{14}$ C]arginine monohydrochloride was obtained from Amersham International. All other compounds were from the Sigma Chemical Company. The data are expressed as the mean  $\pm$  SEM of (n) rats per experimental group. Data were analyzed with the Tukey-Kramer Multiple Comparisons test, where p<0.05 was taken as significant.

#### **Results**

We found that cNOS activity was higher in the aorta and heart of female rats compared to males (by  $105\pm10\%$  and by  $104\pm18\%$ , respectively; n=7-9; p<0.001). Ovariectomy decreased cNOS activity in both tissues to that level what could be observed in males (by  $58\pm6\%$  in the aorta and by  $49\pm4\%$  in the heart; n=7-9; p<0.001). Estrogen supplementation in the ovariectomized rat caused a dose-dependent elevation of cNOS enzyme activity in cardiac and aortic tissues, where the higher dose ( $100~\mu g$ ) completely restored cNOS enzyme activity to that level what could be observed in females. Data are shown in Fig. 1.

In female and male rats, there was no significant gender difference in iNOS activity in the aorta (being  $0.07\pm0.04$  and  $0.09\pm0.04$  pmol/min/mg protein, respectively; n=7-8) and in

plasma concentration of estrogen is high [21], and administration of estraction increased endothelium-dependent arterial relaxation [22]. Our results accord with carlier observations where physiological levels of circulating 17-\(\beta\)-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 [23]. Our observations are in agreement with the earlier findings of Weiner et al. [10], where an increase in endogenous estrogen level (by pregnancy), and administration of exogenous estrogen to intact females elevated the calcium-dependent cNOS enzyme activity and mRNA expression in the uterine artery and heart of guinea pigs. Interestingly, in a recent report of Nuedling et al. [24], 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, which result may provide a further insight for the better understanding of estrogen-mediated cardiac protection. Moreover, NO has been found to be involved in the early and delayed cardiac protective effects of preconditioning induced by coronary artery occlusion [25], cardiac pacing [26,27] and non-toxic endotoxin treatment [27,28] in various species. In addition, estradiol supplementation improved vasodilatation in postmenopausal women, because of augmented NO production [29]. Finally, chronically administered estrogen enhanced the release of NO from the vascular endothelium of the rat nortic ring [30].

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. In summary, it is known that inhibition of cNOS increases blood pressure, platelet aggregation and leukocyte 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 effects of cNOS [8,9]. However, recent studies have shown that NO synthesized in neuronal elements by nNOS might have similar vascular protective actions [9,10] whose activity is also included in cNOS. It seems that further studies are needed to clarify the role of nNOS in cardiovascular protection.

In contrast, NO donors, because of their smooth muscle relaxing effect, are well-known arterial and venous vasodilators. Furthermore, they inhibit leukocyte adherence to the vascular endothelium and platelet aggregation, processes that have crucial role in vascular congestion. It is also well established that NO donors are used worldwide 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 [31]. Thus, NO synthesized by cNOS preserves normal vascular tone, leukocyte deposition and platelet aggregation [8,9].

Taking the results together, it is strongly suggested, that the endogenous estrogens up-regulate cNOS enzyme activity in the rat aorta and heart. This estrogen-mcdiated increase of NO production by cNOS might possibly be an explanation of the sexual dimorphism of ischemic heart disease; i.e. women are less sensitive than men are in the fertile age.

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Nitric oxide-mediated mucus hypersecretion protects the stomach of ovariectomized rats.

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#### Rapid communication

# Nitric oxide-mediated mucus hypersecretion protects the stomach of ovariectomized rats

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#### **Abstract**

The actions of ovariectomy on nitric oxide synthase (assessed by the citrullin assay), mucus secretion (assessed by the Alcyan blue technique) and ulcerogenic response (indomethacin (30 mg kg<sup>-1</sup>, s.c., 4 h) or cysteamine (400 mg kg<sup>-1</sup>, s.c., 24 h)) were studied in the female rat stomach. Ovariectomy increased nitric oxide synthase and mucus secretion, and decreased the severity of lesions, effects reversed by the nitric oxide synthase inhibitor,  $N^G$ -nitro-L-arginine methyl ester (L-NAME, 10 mg kg<sup>-1</sup>, s.c., 4 h before measurements). Therefore, estrogen-deficiency protects the gastric mucosa by nitric oxide (NO)-mediated mucus hypersecretion. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nitric oxide (NO); Gastric mucus; Estrogen

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). 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). Among the protective factors, gastroduodenal mucus plays a crucial role (Robert and Kauffman, 1989). Administration of nitric oxide (NO) donors stimulates mucus release from isolated gastric mucus-cell fraction (but not in parietal cells), and it has also been demonstrated that NO synthase is presented in these cells (Brown et al., 1992, 1993). Moreover, an increase in endogenous NO synthase activity in the gastric mucosa protected the stomach against damage (Tepperman et al., 1993). In the present study, the action of the estrogen-deficient state (i.e. ovariectomy) on gastric NO synthase activity, mucus secretion and on the susceptibility of the mucosa towards various ulcerogenic stimuli has been examined.

We used female Wistar rats (200-220 g). Sham operation or ovariectomy has been performed 1 month before the experiments. For provocation of gastric mucosal injury, indomethacin (30 mg kg<sup>-1</sup>, s.c.) or cysteamine (400 mg kg<sup>-1</sup>, s.c.) were administered, and the measurement (planimetry) of lesions has been performed 4 or 24 h later, respectively. The animals were starved for 24 h before (indomethacin) or during (cysteamine) lesion induction. For the evaluation of gastric mucosal NO synthase activity, we used the citrulline assay. The detailed description of the method can be found in the paper of Brown et al. (1992). For the measurement of gastric mucus level, we used the Alcyan blue technique, which methodological description can be found in details in the paper of Drago et al. (1999). For the investigation of the role of NO in our model, the NO synthase inhibitor, NG-nitro-L-arginine methyl ester (L-NAME, 10 mg kg<sup>-1</sup>, s.c.) was administered into ovariectomized rats 4 h before any measurement. For the citrulline assay, L-[U-14C]arginine monohydrochloride was obtained from Amersham International. All other com-

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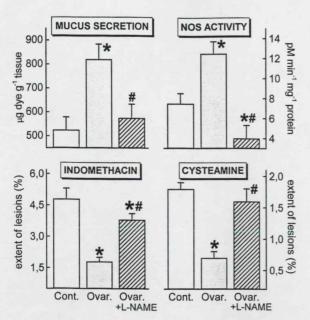


Fig. 1. Actions of ovariectomy (Ovar., grey columns) on mucus secretion (expressed as  $\mu g$  dye  $g^{-1}$  tissue), nitric oxide synthase activity (NOS, expressed as pM min<sup>-1</sup> mg<sup>-1</sup> protein) and lesion formation (expressed as extent of lesions in percent) provoked by indomethacin or cysteamine in the stomach of the female rat (Cont., open columns), and their reversal by the administration of the nitric oxide synthase inhibitor,  $N^G$ -nitro-L-arginine methyl ester (L-NAME, hatched columns). Data are expressed as means  $\pm$  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.

pounds were from Sigma. Data were analyzed with the Turkey-Kramer multiple comparisons test, where P < 0.05 was taken as significant.

We found that ovariectomy increased gastric NO synthase activity (by  $96 \pm 11\%$ , n = 6, P < 0.005) and mucus secretion (by  $57 \pm 8\%$ , n = 5, P < 0.01), and decreased the severity of lesions (by  $63 \pm 10\%$  or by  $61 \pm 6\%$  following indomethacin or cysteamine challenge, respectively; n = 4-5, P < 0.001). All these effects of ovariectomy were reversed by the administration of the NO synthase inhibitor, L-NAME (Fig. 1).

Our present findings confirm that gonadectomy improves the defensive mechanism of the stomach (László et al., 1997; Drago et al., 1999). 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 the gastric protection by ovariectomy and lactation, when the level of estrogens is low (Robert and Kauffman, 1989; Drago 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 their estrogen level is low. However, orchidectomy or testosterone antagonist revealed to protect against injury. This shows that in males, instead of harmful estrogens, testosterone plays an

aggressive role towards the gastric mucosa (László et al., 1997). Testosterone may generate more severe gastric damage in males compared to females, since in males the protective progesterone level is also low.

In our study, an increased gastric mucosal NO synthase activity has been shown after ovariectomy. This elevated gastric NO production might have an important role in the protection against ulcerogenic challenge, since the administration of the NO synthase inhibitor, L-NAME restored mucosal damage in the ovariectomized rat to that level what could be observed in control females. An increased generation of NO may play a role among gastric protective mechanisms by its ability to maintain microvascular integrity (Whittle, 1993), which is also 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 a time-response study, we found that microvascular injury preceded mucosal damage following indomethacin administration, while the opposite could be observed with cysteamine (our unpublished results). In our present study, we found that in ovariectomized rats the mucus secretion in the stomach has been increased, an effect reversed by L-NAME. 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.

In conclusion, estrogen-deficiency provokes a NO-mediated gastric mucus hypersecretion, which improves mucosal defence.

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#### Short communication

# Raloxifene, an oestrogen-receptor modulator, prevents decreased constitutive nitric oxide and vasoconstriction in ovariectomized rats

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#### Abstract

Administration of graded doses of [Arg<sup>8</sup>]vasopressin (0.06–0.18 μg kg<sup>-1</sup>, i.v.) induced a dose-dependent increase in arterial blood pressure in the catecholamine-depleted (phentolamine; 10 mg kg<sup>-1</sup>, i.p.) intact and ovariectomized female rat, with the elevation of blood pressure more marked following ovariectomy. In addition, ovariectomy caused the down-regulation of aortic Ca<sup>2+</sup>-dependent constitutive nitric oxide synthase (assessed by the citrulline assay). The down-regulation of the Ca<sup>2+</sup>-dependent constitutive nitric oxide synthase and augmentation of vasopressin-induced blood pressure responses were prevented by the therapy (1 month, p.o.) with the selective oestrogen receptor modulator, raloxifene (0.3–1.0 mg kg<sup>-1</sup> day<sup>-1</sup>), or with 17β-oestradiol (0.3 mg kg<sup>-1</sup> day<sup>-1</sup>) in ovariectomized rats. Thus, oestrogen deficiency down-regulates vascular constitutive nitric oxide synthase, which appears to be involved in the increased sensitivity of the vasculature to vasopressin, since both effects can be reversed by the exogenous administration of the natural oestrogen 17β-oestradiol or the selective oestrogen-receptor modulator raloxifene. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Oestrogen receptor modulator; Oestrogen; Nitric oxide (NO); Blood pressure; Vasopressin; Enzyme regulation

#### 1. Introduction

In the reproductive years, the incidence of cardiovascular disease is lower among women than among men. This gender difference disappears following either natural or surgical menopause, suggesting a protective role of endogenous estrogens in the cardiovascular system (Clarkson et al., 1997).

Under experimental conditions, endogenous estrogen or administration of natural estrogens has been demonstrated to increase Ca<sup>2+</sup>-dependent constitutive nitric oxide (NO) synthase (cNOS) activity in vascular tissues (Weiner et al., 1994; Morschl et al., 2000). Selective oestrogen-receptor modulators have tissue-specific oestrogen agonist effects, e.g. on bone and lipid metabolism, and antagonist effects, e.g. on breast (Clarkson et al., 1997). In addition, increased

It is known that NO, formed continuously by cNOS in the vascular endothelium and neuronal elements, plays a significant protective role in the maintenance of vascular integrity. The inhibition of cNOS provokes blood pressure elevation, platelet aggregation and adhesion of neutrophils to the vascular endothelium (Moncada and Higgs, 1995). Moreover, it has recently been demonstrated that oestrogen can modulate the action of vasopressin on the baroreflex control of sympathetic outflow, and thereby participate in cardiovascular regulation (He et al., 1999).

In the present study, we investigated the vasopressin-induced blood pressure response in catecholamine-depleted ovariectomized rats, by determining the changes of aortic cNOS enzyme activity in conjunction with the vasopressin-provoked blood pressure response following raloxifene or oestrogen supplementation in the oestrogen-deficient state.

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).

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#### 2. Materials and methods

#### 2.1. Experimental protocol

We used sham-operated and ovariectomized female Wistar rats (200–220 g). Operations were performed under transient ether anaesthesia, followed by a 1-month recovery period. In ovariectomized rats, raloxifene (0.3–1 mg kg<sup>-1</sup>, p.o., once daily) or 17β-oestradiol (0.3 mg kg<sup>-1</sup>, p.o., once daily) were administered for 1 month. The procedures, doses of compounds, and route of administration have been established in previous studies (Rahimian et al., 1997; Morschl et al., 2000). Control animals received the vehicle at the same time, in the same volume and by the same route.

# 2.2. Ca<sup>2+</sup>-dependent aortic nitric oxide synthase enzyme activity

NOS activity was determined as the conversion of L-[14Clarginine monohydrochloride to L-[14Clcitrulline based on the method described previously (Salter et al., 1991), with minor modifications aiming to detect the activity of cNOS (Weiner et al, 1994; Garvey et al., 1997). We sacrificed the animals by decapitation, and immediately after autopsy prepared fresh tissues for NOS measurements. Aortic tissues were pooled from the abdominal aorta of two rats. Tissues were homogenized (15 s, Ultra-Turrax homogenizer, 5 mm blade) in buffer (250 mg ml<sup>-1</sup>, 4°C, 10 mM HEPES, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 10 µg ml<sup>-1</sup> soybean trypsin inhibitor, 10 μg ml<sup>-1</sup> leupeptin, 2 μg ml<sup>-1</sup> aprotonin, pH 7.4). This was followed by centrifugation (20 min,  $10,000 \times g$ , 4°C). Samples were mixed with Dowex (AG 50W-8; 200-400, 8% cross-linked, Na form) resin. After further centrifugation (10 min,  $10.000 \times g$ , 4°C), the supernatant (40  $\mu$ l) was incubated for 10 min at 37°C in reaction buffer comprising (final concentrations): 50 mM KH, PO4, 10 µg ml-1 calmodulin, 2.5 mM CaCI<sub>2</sub>, 50 mM valine, 1 mM dithiothreitol, 15.5 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 µM flavin adenine dinucleotide (FAD), 3 μM flavin mononucleotide (FMN), 3 μM tetrahydrobiopterin and 0.17 µM of [14C]L-arginine. The reaction was terminated 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 by spectrophotometric assay (Bio-Rad Protein Assay), and NOS activity expressed as pmol min-1 mg-1 protein, with constant linear rates for different amounts of extracts.

Total NOS activity was defined as citrulline formation 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  $Ca^{2+}$ -dependent cNOS

activity. In addition, Ca<sup>3+</sup>-independent NOS activity (iNOS) was also determined as the difference between samples containing 1 mM EGTA and samples containing 1 mM L-NNA.

#### 2.3. Blood pressure response to vasopressin

Animals were anaesthetised with urethane (1.25 g kg<sup>-1</sup>, i.p.), and then pre-treated with phentolamine (10 mg kg<sup>-1</sup>, i.p.). A single bolus injection of arginine-vasopressin (0.06-0.18 µg kg<sup>-1</sup>, i.v.) was administered into the tail vein following the stabilisation of blood pressure. The procedure in details has been described previously (László et al., 1991). Briefly, the elevation of blood pressure (expressed as a % maximal increase compared to the basal value) was measured in the right carotid artery through a blood pressure transducer connected to the HAEMOSYS computerised complex haemodynamic analysis system (Experimetria UK, London). The core temperature of rats was maintained at 37°C with a homeothermic control unit (Harvard Instrument, UK).

#### 2.4. Chemicals

L-[14C]arginine monohydrochloride, raloxifene, arginine-vasopressin and phentolamine were purchased from Amersham International (UK), Eli Lilly and Company (USA), Organon OSS (The Netherlands) and Ciba-Geigy (Switzerland), respectively. All non-specified agents were from Sigma.

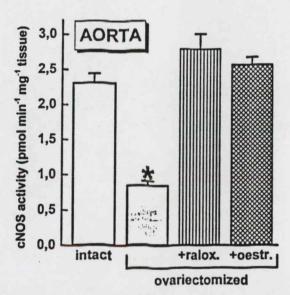


Fig. 1. Ovariectomy-provoked decrease in constitutive aortic nitric oxide synthase (cNOS) enzyme activity (expressed as pmol min<sup>-1</sup> mg<sup>-1</sup> tissue). Actions of raloxifene (ralox.; 1 mg kg<sup>-1</sup>) or 17 $\beta$ -oestradiol (oestr.: 0.3 mg kg<sup>-1</sup>) supplementation (1 month, p.o.) on cNOS enzyme activity in the aorta of ovariectomized rats. Data are expressed as mean  $\pm$  S.E.M. of five measurements in each group; \*P < 0.001 means significant difference between the intact female and the ovariectomized group.

#### 2.5. Statistics

The data are expressed as the mean  $\pm$  S.E.M. of (n) rats per experimental group. Data were analyzed by the Tukey-Kramer Multiple Comparisons test, with P < 0.05 taken as significant.

#### 3. Results

# 3.1. Ca<sup>2+</sup>-dependent aortic nitric oxide synthase enzyme activity

As shown in Fig. 1, ovariectomy significantly decreases aortic cNOS activity, i.e. it falls to  $37 \pm 2\%$  of the intact rat (n = 5; P < 0.001). In the aorta of the ovariectomized rat,  $17\beta$ -oestradiol (0.3 mg kg<sup>-1</sup>) or raloxifene (1 mg kg<sup>-1</sup>) supplementation (1 month, p.o.) completely restored cNOS enzyme activity to levels seen in intact females.

In female rats, iNOS activity in the aorta was  $0.08 \pm 0.05$  pmol min<sup>-1</sup> mg<sup>-1</sup> protein (n = 5). Neither ovariectomy nor administration of  $17\beta$ -oestradiol or raloxifene changed aortic iNOS activities compared to the intact basal values (n = 5, data not shown).

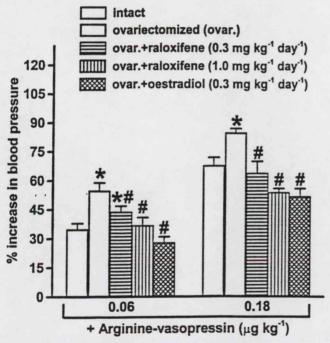


Fig. 2. Increase of arterial blood pressure (expressed as % change, measured in the right carotid artery) by intravenous administration of arginine-vasopressin (0.06–0.18  $\mu$ g kg<sup>-1</sup>) in the catecholamine-depleted (phentolamine, 10 mg kg<sup>-1</sup>, i.p.) sham-operated and ovariectomized (1 month) female rat. Effect of supplementation (1 month, p.o.) with 17 $\beta$ -oestradiol (0.3 mg kg<sup>-1</sup> day<sup>-1</sup>) or the selective oestrogen-receptor modulator, raloxifene (0.3–1 mg kg<sup>-1</sup> day<sup>-1</sup>) on vasopressin-induced blood pressure elevation in the ovariectomized rat. Data are expressed as mean  $\pm$  S.E.M., where n = 8-12 rats in a group. \*P < 0.05 means significant blood pressure increase compared to the vasopressin-treated intact female group; \*P < 0.05 means significant blood pressure decrease compared to the vasopressin-treated ovariectomized female group.

#### 3.2. Blood pressure response to vasepressin

As shown in Fig. 2, administration of vasopressin caused a dose-dependent increase of arterial blood pressure both in the ovary-intact and ovarjectomized female rat. However, in the ovariectomized animals, vasopressin induced a significantly higher elevation of blood pressure than in ovary-intact females. Oestrogen replacement abolished the increased blood pressure response observed, and raloxifene supplementation caused a dose dependent decrease in blood pressure enhancement provoked by vasopressin in ovariectomized rats.

#### 4. Discussion

The present findings are in agreement with recent observations that vasoconstrictor tone is increased in various vascular beds following ovariectomy, an effect that can be mitigated by oestrogen 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 oestrogen-deficient state.

Under in vitro circumstances, a reduction of the basal release of NO, as detected by augmented catecholamineinduced contractile response in the presence of an NO synthase inhibitor, was found in nortic rings from ovariectomized animals, an effect blocked by raloxifene or oestradiol treatment (Rahimian et al., 1997). Under in vivo conditions, reduced cNOS activity has been demonstrated in the aortic tissue of ovariectomized rats compared with normal females, and administration of 17β-oestradiol dose-dependently increased aortic cNOS activity in ovariectomized rats to normal female levels (Morschl et al., 2000). Our present findings give further support to the proposition that vascular cNOS activity is regulated by estrogen, and that under in vivo circumstances raloxifene has oestrogen agonist properties with respect to vascular cNOS activity.

Finally, 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 vasopressin. 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 cNOS 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): it effectively counteracts the increase in vascular permeability provoked by 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 vasopressin in the absence of oestrogen.

In conclusion, our results suggest that in oestrogen-deficiency aortic cNOS is down-regulated, which strongly suggests its involvement in the increased sensitivity of the vasculature to the vasoconstrictor effect of vasopressin. Both actions can be reversed by the therapy with the natural oestrogen 17β-oestradiol, or the selective oestrogen-receptor modulator raloxifene. Thus, raloxifene behaves as an oestrogen receptor agonist with regards to both regulation of vascular cNOS and vasopressin-provoked increases in blood pressure in vivo.

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Vasopressin deficiency decreases the frequency of gastroduodenal ulceration in humans.

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## Vasopressin deficiency decreases the frequency of gastroduodenal ulceration in humans

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Abstract — Vasopressin is a stress hormone released from the posterior pituitary. In humans suffering from central flabetes insipidus, this release of vasopressin is diminished. It was shown previously that the congenutally vasopressin-deficient Brattlehorn homozygous rat is less sensitive to various ulcerogenic stimuli. In this study, we investigated the incidence of gastroductional ulceration in vasopressin deficient patients. Data on patients aged 20–70, hospitalized in Hungary between 1992 and 1995 were compared with those on the total population in this age group (6 681 020 in 1994). Subjects with central diabetes insipidus were selected separately (815 cases). Gastroducdenal ulceration was compared in subjects with an intact vasopressin release and vasopressin-deficient patients. The frequencies of gastroducdenal ulceration were also examined separately in male and female subjects. In the total population, the frequency of gastroducdenal ulceration was lower in vasopressin-deficient cases (2.22% versus 0.61%; P < 0.005). Among normal-vasopressin subjects, males have a higher risk of gastroducdenal ulceration than females (3.04% versus 1.46%, respectively; P < 0.001). Among vasopressin-deficient subjects, a similar male:female ratio was observed, but it was not significant (P = 0.36). In comparison to the normal-vasopressin population, the incidence of gastroducdenal ulceration was reduced among vasopressin-deficient males and females by 77% (P < 0.01) and by 82% (P < 0.05), respectively. In conclusion, endogenous vasopressin has a significant harmful action towards the human gastroducdenal mucosa. Peptide and non-peptide vasopressin receptor antagonists might have a potential therapeutic benefit in the treatment (as an adjuvant) and prevention of gastroducdenal ulceration. © 2000 Plasevier Science Ltd. Published by Editions scientifiques et médicales Elsevier SAS

gastric ulcer / duodenal ulcer / ulcer disease / vasopressin / vasopressin receptor antagonist / diabetes insipidus

#### 1. Introduction

Gastroduodenal ulceration is a common disease. It is known that aggressive (e.g., gastric acid, pepsin) and protective (e.g., mucosal blood flow, prostaglandins) factors act in the development of acute or chronic mucosal lesions, and an imbalance between them plays a key role in the occurrence of the disease [15].

In animals, endogenous vasopressin has been demonstrated to act as an aggressive factor towards the gastroduodenal mucosa, since the vasopressin-deficient Brattleboro homozygous rat [16] is less sensitive to various ulcerogenic stimuli than the rat with normal-vasopressin secretion [5, 6, 8].

In humans, suffering from central diabetes insipidus, the secretion/release of vasopressin from the hypothalamus/posterior pituitary is impaired. These patients are ideal subjects of the investigation of the role of endogenous vasopressin in generation of various diseases, in which this stress hormone might possibly have a pathogenetic action. Accordingly the aim of the present study was to evaluate the incidence of gastroduodenal ulceration among vasopressin-

deficient human subjects in comparison to the normal-vasopressin population.

#### 2. Materials and methods

#### 2.1. Study groups

Two groups of human subjects aged between 20 and 70 years were selected: the vasopressin-deficient and the non-deficient groups. The vasopressin-deficient cases suffered from central diabetes insipidus, while the non-deficients were taken as the normal population in Hungary in 1994 (data on 01.01.1995). All central diabetes insipidus cases hospitalized in Hungary between 01.01.1992 and 01.10.1995 were selected. Additionally, the subjects in the two groups were examined on the basis of their sex.

#### 2.2. Cases with gastroduodenal ulceration

All patients aged 20-70 years hospitalized in Hungary between 01.01.1992 and 01.10.1995 with gastroduodenal ulceration (including acute or chronic gastric and/or duodenal ulceration with or without

<sup>\*</sup> Correspondence and reprints

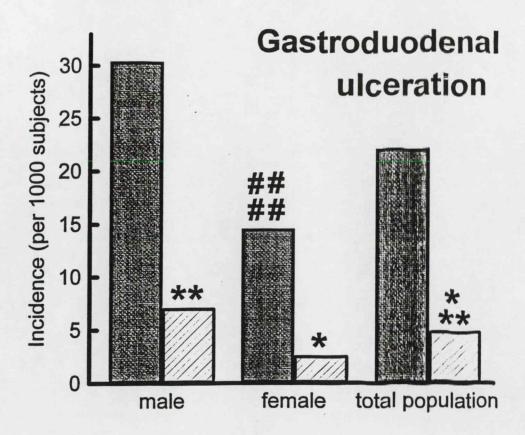


Figure 1. Incidence (per 1 000 subjects) of gastroduodenal ulceration (including acute and chronic gastric and/or duodenal ulceration with or without complication, such as bleeding and/or perforation) among the normal Hungarian population aged 20–70 years (males, females and total population) with an intact vasopressin release (gray column) or with a vasopressin deficiency (hatched column). Statistical significance: \*P < 0.05; \*\*P < 0.01; and \*\*\*P < 0.005 for normal-vasopressin versus vasopressin-deficient cases, and \*\*\*\*P < 0.001 for males versus females.

12], may generate vasoconstriction and platelet aggregation (via its V<sub>1</sub> receptors), and release coagulant factors (via its antidiuretic V<sub>2</sub> receptors), leading to a reduced blood flow and a consequent hypoxia in the gastroduodenal mucosa. The reduction of mucosal oxigenation might attenuate the protective mechanisms of the gut and sensitize it to the damaging effect of other well-known aggressive factors, such as gastric acid and pepsin [15]. Indeed, administration of vasopressin V<sub>1</sub> receptor antagonists elevates blood flow in the superior mesenteric artery [14] and protects against various ulcerogenic challenge [5, 6, 8, 9]. Although in a previous study, a selective V<sub>2</sub> receptor antagonist has been reported to be ineffective against ethanol-induced gastric hemorrhagic erosions in the rat [6], but the involvement of this type of vasopressin receptor seems not to be excluded in generation of gastroduodenal ulceration, since a non-selective vasopressin receptor antagonist ( $V_1/V_2$  antagonist) was shown to be more potent in the ethanol model than the selective  $V_1$  receptor antagonist alone (our unpublished result).

In conclusion, endogenous vasopressin seems to have a significant harmful action towards the human mucosa in generation of gastroduodenal ulceration. Peptide and orally effective non-peptide vasopressin receptor antagonists may have a potential therapeutic benefit in the prevention and treatment (as an adjuvant) of gastroduodenal ulceration.

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#### Short communication

## Interactions of pro-inflammatory and vasoactive mediators with nitric oxide in the regulation of rat vascular permeability during laparotomy

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#### Abstract

Inhibition of constitutive nitric oxide (NO) synthases by administration of  $N^G$ -nitro-L-arginine methyl ester (1.-NAME) during abdominal laparotomy provokes extensive vascular leakage in the rat gastrointestinal tract, assessed by the extravasation of [123] human serum albumin. In the present study, the role of vasoactive or neutrophil-derived pro-inflammatory mediators in this process has been investigated. Administration of the thromboxane synthase inhibitor, 1-benzyl-imidazole (BZI, 25–50 mg kg<sup>-1</sup>, s.c.), the platelet-activating factor (PAF) receptor antagonist, 3-[4-(2-chlorophenyl)-9-methyl-6H-thienol-[3,2-f][1,2,4]-triazolo-[4,3-a][1,4]-diazepine-2-yl]-1-(4-morpholynil)-1-propionate (WEB 2086; 0.5–1 mg kg<sup>-1</sup>, s.c.), the 5-lipoxygenase synthase inhibitor, N-(4-benzyloxybenzyl)-acetohydroxamic acid (BW A137C; 4–20 mg kg<sup>-1</sup>, s.c.) or the vasopressin pressor receptor antagonist ([Mca<sup>1</sup>,Tyr(Me)<sup>2</sup>,Arg<sup>3</sup>]vasopressin/Manning peptide; 0.01–0.2 µg kg<sup>-1</sup>, s.c.) dose-dependently reduced the intestinal plasma leakage provoked by L-NAME (5 mg kg<sup>-1</sup>, s.c.), following a 5-cm abdominal laparotomy in anaesthetised rats. These findings suggest that constitutive NO synthase effectively counteracts the damaging actions on microvascular integrity of mediators, including thromboxanes, PAF, leukotricnes and vasopressin, released during surgical intervention. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Nitric oxide (NO); Vascular permeability; Vascular endothelium; Neutrophil granulocytes; Platelet-activating factor (PAP); Thromboxanes; Leukotrienes; Vasopressin; Surgery

#### 1. Introduction

During major surgical operations, a reduction in circulating plasma volume can occur, following increased plasma extravasation, which develops without significant bleeding (Krakelund, 1971; Akerström and Lisander, 1991). However, few experimental or clinical studies have explored the mechanism underlying this phenomenon (Akerström and Lisander, 1991; László and Whittle, 1999; László et al., 1999).

Nitric oxide (NO), formed continuously in the vascular endothelium by the constitutive NO synthase, eNOS, plays

a key role in the maintenance of vascular integrity (Moncada and Higgs, 1995). NO attenuates the adherence and immigration of leukocytes in the vascular endothelium (Kubes et al., 1991), a process of key importance in the generation of vascular endothelial dysfunction, vascular congestion and inflammation (Wedmore and Williams, 1981). Indeed, inhibition of the constitutive NO synthase, eNOS, leads to neutrophil adhesion and the elevation of vascular permeability, particularly in surgically prepared models (Kubes et al., 1991; Kubes and Granger, 1992; Arndt et al., 1993).

In recent studies, constitutively formed NO has been suggested to play a beneficial role against widespread plasma loss, observed during surgical operation, through the maintenance of microvascular integrity (László and Whittle, 1999; László et al., 1999). Thus, midline abdominal laparotomy and anaesthesia alone, or administration of

endothelium by the constitutive NO synthase, eNOS, plays

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the NO synthase inhibitor, N<sup>G</sup>-nitro-L-arginine methyl ester (L-NAME), to anaesthetised animals alone, did not affect vascular permeability. In contrast, when L-NAME was administered to laparotomized animals, a generalised increase of albumin leakage developed in intestinal organs as well as in the lungs and kidneys (László and Whittle, 1999). A reduction in the number of circulating neutrophils attenuated this vascular leakage provoked by L-NAME and laparotomy in all of the organs investigated, which suggests an involvement of neutrophil-derived pro-inflammatory mediators in this surgical plasma extravasation (László and Whittle, 1999).

In previous studies, a role for the neutrophil-derived pro-inflammatory mediators thromboxanes, platelet-activating factor (PAF) and leukotrienes, on the development of vascular leakage provoked by the acute administration of L-NAME following bacterial endotoxin challenge, has been established (László and Whittle, 1995; László et al., 1994). In the present work, the involvement of such mediators in responding to L-NAME following midline abdominal laparotomy has been explored using specific antagonists and synthase inhibitors. In addition, using a specific vasopressin pressor receptor antagonist, the actions of endogenous vasopressin, which is known to be released during surgical interventions (Melville et al., 1985), have been evaluated.

#### 2. Materials and methods

#### 2.1. Surgical manipulation

Male Wistar rats (225–275 g) were starved overnight, but were allowed free access to water. In the basal control group, the treatments were performed under transient halothane anaesthesia from which the animals had completely recovered within 2 min. Autopsy in this surgically unoperated group was performed under halothane anaesthesia. In all other groups (using pentobarbitone-anaesthetised animals), a 5-cm long midline laparotomy in the abdominal wall was performed without significant bleeding. Rats were tracheotomized and a gauze pad moistened with saline was placed over the incision. At the beginning of the experiment, all groups were administered [ $^{125}$ I]human serum albumin (2  $\mu$ Ci kg $^{-1}$ , i.v.) via a needle inserted into the tail vein for 3–4 s. Autopsy was performed 1 h later.

#### 2.2. Plasma leakage

As a measure of vascular endothelial permeability, leakage of [125] human serum albumin into tissue was determined in segments of the jejunum and colon. Blood were collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0.318%) and were

centrifuged (10,000 × g. 10 min. 4°C). The [125] human serum albumin content of the plasma and of segments of tissues was detected in a gamma spectrometer (Nuclear Enterprises NE 1600), and the albumin content in tissues was calculated.

The resting value for albumin accumulation was taken as the mean of the data of a group of basal control animals. In each experiment, and for each procedure, this basal control mean value was calculated and subtracted from the value from each of the animals in each treatment group. The data were expressed as changes in albumin accumulation (Aplasma leakage, in microliter plasma per gram tissue) corrected for intravascular volume (László et al., 1994).

#### 2.2.1. Intravascular volume

Changes in intravascular volume in jejunal and colonic tissues were determined in additional groups of rats by administering [123]]human perum albumin (2 µCi kg<sup>-1</sup>) intravenously via the tail vein 2 min before tissue removal, in all groups investigated. The tissue and plasma content of radiolabel were determined and the intravascular volume was expressed as microliter per gram tissue (László et al., 1994).

### 2.3. Effect of L-NAME and laparotomy on intestinal plasma leakage

In groups of rats, L-NAME (5 mg kg<sup>-1</sup>, s.c.) was injected concurrently with [<sup>125</sup>I]human serum albumin. Plasma leakage in the jejunum and colon was evaluated after 1 h. The dose of L-NAME and the timing of the experiment were based on the results of our previous study (László and Whittle, 1999).

## 2.4. Effect of pro-inflammatory and vasoactive mediators on plasma leakage provoked by L-NAME and laparotomy

In laparotomized and L-NAME (5 mg kg<sup>-1</sup>, s.c.)-treated rats, the thromboxane synthase inhibitor, 1-benzyl-imidazole (BZI; 25-50 mg kg<sup>-1</sup>), the PAF receptor antagonist, (3-[4-(2-chlorophenyl)-9-methyl-6 H-thienol-[3, 2-f[[1,2,4]-triazolo-[4,3-u][1,4]-diazepine-2-yl]-1-(4-morpholynil)-1-propionate (WEB 2086; 0.5-1 mg kg<sup>-1</sup>), the 5-lipoxygenase inhibitor, N-(4-benzyloxybenzyl)-acetohydroxamic acid (BW A137C; 4-20 mg kg<sup>-1</sup>) or the vasopressin pressor receptor antagonist ([Mca<sup>1</sup>,Tyr(Me)<sup>2</sup>, Arg<sup>8</sup>]vasopressin/Manning peptide; 0.01-0.2 µg kg<sup>-1</sup>) was injected s.c., 15 min before laparotomy and L-NAME administration. Plasma leakage in jejunal and colonic tissues was determined 1 h after laparotomy. All the compounds were dissolved in saline immediately before the experimentation. The doses, timing and route of administration of drugs were established in our previous studies

(László et al., 1991, 1994; László and Whittle, 1995, 1999).

#### 2.5. Chemicals

[125] I]human serum albumin was obtained from Amersham International (UK). BZI and BW A137C originated from the Wellcome Research Laboratories, Beckenham, UK. WEB 2086 was supplied by the Boehringer Ingelheim, Germany. The vasopressin antagonist was acquired from Bachem, Germany. All the other compounds were from Sigma (Poole, Dorset, UK).

#### 2.6. Statistics

The data are expressed as mean  $\pm$  SEM from (n) rats per experimental group. For statistical comparisons, analysis of variance with the Bonferroni test was utilised, where P < 0.05 was taken as significant.

#### 3. Results

## 3.1. Intestinal albumin leakage provoked by L-NAME during laparotomy

No significant intestinal albumin leakage could be observed when L-NAME (5 mg kg<sup>-1</sup>, s.c.) was administered alone into the anaesthesized non-laparotomized rat after 1 h ( $\Delta 2 \pm 4$  and  $\Delta 6 \pm 3$   $\mu$ l g<sup>-1</sup> tissue in the jejunum and

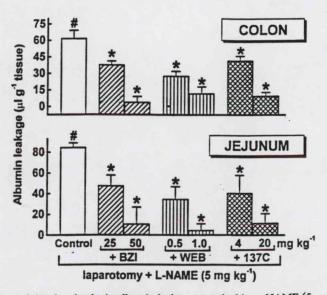


Fig. 1. Jejunal and colonic albumin leakage provoked by L-NAME (5 mg kg<sup>-1</sup>, s.c.) in the laparotomized rat over 1 h. The effects of pretreatment (15 min) with a thromboxane synthase inhibitor (BZI, 25–50 mg kg<sup>-1</sup>, s.c.), with a PAF receptor antagonist, WEB 2086 (WEB, 0.5–1.0 mg kg<sup>-1</sup>, s.c.), or with a leukotriene synthase inhibitor, BW A137C (137C, 4–20 mg kg<sup>-1</sup>, s.c.) are shown. Data are expressed as the mean  $\pm$  SEM, where (n) is minimum five rats for each group, and where statistical significance is given as \*P < 0.05 compared to conscious untreated animals; \*P < 0.05 compared to L-NAME plus laparotomy (control) groups.

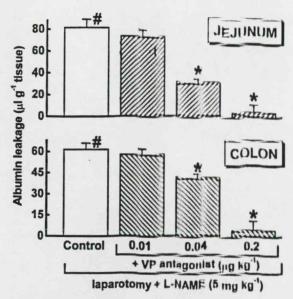


Fig. 2. Dose-dependent attenuation of L-NAMI: (5 mg kg<sup>-1</sup>, s.c.)-provoked rat jejunal and colonic albumin leakage in laparotomized rats over 1 h, by the pretreatment (15 min) with the vasopressin pressor receptor antagonist (VP antagonist,  $0.01-0.2~\mu g~kg^{-1}$ , s.c.). Data are expressed as the mean  $\pm$  SEM, where (n) is minimum five rats for each group, and where statistical significance is given as  $^{\circ}P < 0.05$  compared to conscious untreated animals;  $^{\circ}P < 0.05$  compared to L-NAME plus laparotomy (control) groups.

colon, respectively; n=7). Laparotomy alone did not provoke significant jejunal or colonic albumin leakage over the 1-h study period ( $\Delta 5 \pm 7$  and  $\Delta 1 \pm 7$   $\mu 1$  g<sup>-1</sup> tissue in the jejunum and colon, respectively; n=8). In contrast, when L-NAME was administered concurrently with the laparotomy procedures, significant albumin leakage occurred in jejunal and colonic tissues ( $\Delta 82 \pm 3$  and  $\Delta 61 \pm 5$   $\mu 1$  g<sup>-1</sup> tissue, respectively; n=9, P<0.005), as demonstrated in Fig. 1.

## 3.2. Role of pro-inflammatory and vasoactive mediators in intestinal plasma leakage provoked by L-NAME and laparotomy

Pretreatment (15 min) with the thromboxane synthase inhibitor (BZI, 25-50 mg kg<sup>-1</sup>, s.c.), the PAF receptor antagonist (WEB 2086, 0.5-1 mg kg<sup>-1</sup>, s.c.) or a 5-lipoxygenase inhibitor (BW A137C, 4-20 mg kg<sup>-1</sup>, s.c.) dose-dependently attenuated the subsequent jejunal and colonic plasma leakage provoked by laparatomy and L-NAME, with a maximum reduction of  $88 \pm 15\%$ ,  $97 \pm 2\%$  or  $87 \pm 6\%$  respectively in the jejunum (n = 5-7; P < 0.001), as shown in Fig. 1.

Administration of the vasopressin pressor receptor antagonist,  $[Mca^1, Tyr(Me)^2, Arg^8]$ vasopressin (0.01–0.2 µg kg<sup>-1</sup>, s.c., 15 min before laparotomy and L-NAME), decreased jejunal and colonic albumin extravasation induced by L-NAME in the laparotomized rat, with a maximum reduction of  $98 \pm 1\%$  in the jejunum (n - 6; P < 0.001), as shown in Fig. 2.

#### 4. Discussion

In the present study, administration of the NO synthase inhibitor, L-NAME provoked albumin extravasation in the rat intestinal tract following laparotomy, confirming previous observations (László and Whittle, 1999; László et al., 1999). Other workers had earlier demonstrated that L-NAME could induce an increase in vascular permeability in surgically manipulated models (Kubes and Granger, 1992; Filep and Földes-Filep, 1993). These data show that constitutive NO plays a significant beneficial role in the maintenance of microvascular integrity during and following such surgical operations.

Inhibition of NO synthase enhances the adhesion of leukocytes to the vascular endothelium in surgically prepared vascular beds (Kubes et al., 1991; Arndt et al., 1993). Adhesion of polymorphonuclear leukocytes to the vascular endothelium is well-established to play a crucial role in the increase of microvascular permeability (Wedmore and Williams, 1981; Bone, 1991), most likely by releasing neutrophil-derived pro-inflammatory mediators, such as thromboxanes, PAF and leukotrienes (Bone, 1991). Indeed, previous studies have demonstrated that administration of PAF receptor antagonists decreased L-NAME-induced vascular permeability in the post-operative period (Filep and Földes-Filep, 1993). Furthermore, treatment with 5-lipoxygenase inhibitors or PAF receptor antagonists attenuated L-NAME-provoked enhancement of adhesion of neutrophils to the vascular endothelium following surgical preparation (Arndt et al., 1993).

In previous studies, L-NAME has also been shown to enhance substantially the vascular leakage following the acute administration of bacterial endotoxin (László et al., 1994). This acute response was attenuated by PAF receptor antagonists, thromboxane synthase inhibitors or a 5-lipoxygenase inhibitor, BW A137C, suggesting a role for these neutrophil-derived mediators in this process (László et al., 1994; László and Whittle, 1995). Likewise, in the present study, the PAF receptor antagonist, WEB 2086, the thromboxane synthase inhibitor, BZI, or the 5-lipoxygenase inhibitor, BW A137C, significantly attenuated albumin extravasation. Thus, the release of the neutrophil-derived pro-inflammatory mediators PAF, thromboxanes and leukotrienes appears to participate in the increase of intestinal vascular permeability observed as a consequence of acute abdominal surgery following administration of L-NAME...

The release of the pituitary nonapeptide, vasopressin, into the general circulation during surgical operations has been described (Melville et al., 1985). It has been known that vasopressin can be released from the vascular endothelium (Burnstock, 1991). In addition, pathological levels of vasopressin can provoke vascular endothelial dysfunction via its pressor receptors (László et al., 1991). In the present study, administration of the vasopressin pressor receptor antagonist dose-dependently attenuated albumin leakage

provoked by L-NAME in the laparotomized rat. Thus, endogenous vasopressin may also play a role in the development of the enhanced microvascular permeability during surgical intervention.

Our current studies suggest that the regulation of the maintenance of microvascular integrity during surgical operations involves complex interactions between endothelium- and neutrophil-dependent mechanisms. Thus, constitutively formed NO, most likely of endothelial origin, appears to play a significant pathophysiological role in preventing albumin extravasation in response to surgical challenge. Injurious mediators, such as the neutrophil-derived pro-inflammatory thromboxanes, PAF and leukotrienes, appear to be released following laparotomy, as does vasopressin; the latter of which may originate from the posterior pituitary or from the vascular endothelium. The present findings suggest that such mediators may interact either synergistically or in a sequential cascade to provoke the eventual microvascular dysfunction. The processes that stimulate such mediator release following minor surgery are as yet unknown, although the present work suggests that the release or actions of these factors can be modulated by endogenous NO.

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#### Short communication

### Helicobacter pylori lipopolysaccharide-provoked injury to rat gastroduodenal microvasculature involves inducible nitric oxide synthase

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#### Abstract

The actions of a purified *Helicobacter pylori* lipopolysaccharide (3 mg kg<sup>-1</sup>, i.v.) on rat gastric antral and duodenal microvascular integrity (determined as radiolabelled albumin leakage) and the expression of the inducible nitric oxide (NO) synthase (iNOS; assessed by the citrulline assay) were investigated 4 h after challenge. Significant increases of albumin leakage and expression of iNOS in both antral and duodenal tissues were observed following challenge. Concurrent administration of the selective iNOS inhibitor, 1400W (*N*-(8-(aminomethyl)benzyl)-acetamidine; 0.2–1 mg kg<sup>-1</sup>, s.c.), with lipopolysaccharide, caused a dose-dependent attenuation of the gastric and duodenal albumin leakage. Thus, *H. pylori* lipopolysaccharide can initiate the expression of iNOS in the stomach and duodenum following systemic challenge, which can provoke gastroduodenal microvascular dysfunction. © 2001 Published by Elsevier Science B.V.

Keywords: Helicobacter pylori; Lipopolysaccharide; Gastritis; Duodenum; Inflammation; Nitric oxide (NO) synthase inhibitor; Nitric oxide (NO) synthase, inducible

#### 1. Introduction

The acid-resistant Gram-negative bacterium, *Helicobacter pylori*, grows predominantly in the antral region of the stomach and at sites of gastric metaplasia in the duodenum (Blaser, 1990). The pathological involvement of *H. pylori* is recognized in gastric mucosal inflammation and in gastroduodenal peptic ulceration (NIH, 1994). To understand the mechanisms that are in involved in such gastroduodenal tissue inflammation and injury, it is important to evaluate the effects of the soluble mediators produced by *H. pylori* and their actions in promoting cytotoxicity.

The purification of lipopolysaccharide from *H. pylori* has provided an important tool for exploring the cytotoxic processes related to the bacterium and its products (Moran,

1999). One such potential mechanism involves the inappropriate production of nitric oxide (NO), since the expression of the inducible NO synthase enzyme (iNOS) can be 71 triggered by the lipopolysaccharide component of bacterial 72 endotoxins, as well as the cytokines produced by lipopoly-73 saccharide challenge (Moncada and Higgs, 1995; Perez-Perez et al., 1995). This overproduction of NO has cytotoxic potential and provokes widespread microvascular inflammatory reactions with albumin leakage into the interstitium (László et al., 1995). Recent studies have shown that the purified lipopolysaccharide from H. pylori can 79 cause cytotoxicity in rat duodenal epithelial cells following in vivo challenge (Lamarque et al., 2000). Pharmacological studies indicate that this cytotoxic action involves the expression of iNOS in the epithelial cells and the production of superoxide (Lamarque et al., 2000), thus implicating a role of the cytotoxic species, peroxynitrite (Beckman et al., 1990).

In our present study, we have investigated whether 87 administration of a purified *H. pylori* lipopolysaccharide

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leads to the expression of iNOS, determined by the citrulline assay, and the production of microvascular dysfunction, determined as the leakage of radiolabelled albumin, in both the gastric antrum and duodenum of the rat. The association of such microvascular injury with iNOS expression was explored by the use of the bisisothiourea derivative, 1400W (Garvey et al., 1997; László and Whittle, 1997), a potent and selective inhibitor of the iNOS

#### 2. Materials and methods

#### 2.1. Experimental procedures

Male Wistar rats (230-250 g) were fasted overnight but were allowed free access to water. Under transient ether 106 107 anaesthesia, purified H. pylori lipopolysaccharide (3 mg 108 kg<sup>-1</sup>) was administered into the tail vein, and the gastric antrum and the duodenum were removed 4 h later.

#### 2.2. Albumin leakage

For the measurement of vascular permeability, [125]human serum albumin was administered (2 μCi kg<sup>-1</sup>, i.v.) and its leakage into the tissue determined in segments of the gastric antrum and duodenum, 4 h after lipopolysaccharide administration. Blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0.318%) and centrifuged (10,000 × g, min, 4°C). The [125 I]human serum albumin in the plasma and segments of tissues was detected using a gamma-spectrometer (Nuclear Enterprises NE 1600). Subsequently, the tissues were dried at 80°C over 48 h and the 123 124 albumin content on a weight basis was calculated.

The resting value for albumin leakage was taken as the 126 mean of the data of untreated control animals. In each experiment and for each procedure, this basal control mean value was subtracted from the values of treatment groups. The data were expressed as changes in albumin accumula-130 tion (Δ plasma leakage, μl plasma g<sup>-1</sup> dry tissue) as described previously (László et al., 1994, 1995).

#### 2.3. Nitric oxide synthase enzyme activity

NO synthase (NOS) activity was determined as the 135 136 conversion of L-[14C]arginine monohydrochloride to L-C]citrulline (László et al., 1995). After lipopolysaccharide administration (4 h), gastric antral and duodenal tissues were homogenized (15 s) in buffer (250 mg ml<sup>-1</sup>, 140 4°C) containing HEPES (10 mM), sucrose (32 mM), dithiothreitol (1 mM), EDTA (0.1 mM), soybean trypsin 142 inhibitor (10 μg ml<sup>-1</sup>), leupeptin (10 μg ml<sup>-1</sup>) and aprotonin (2 µg ml<sup>-1</sup>). This tissue-containing buffer, adjusted to pH 7.4, was centrifuged at 10,000 × g for 20 min 144 at 4°C. A 40-µl sample of supernatant was incubated for 145 10 min at 37°C in 110 µl of reaction buffer comprising 146 (final concentrations): 50 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 0.2 mM CaCI<sub>2</sub>, 50 mM valine, 1 mM dithiothreitol, 15 nM L-arginine, 1 mM L-citrulline, 0.3 mM NADPH, 3 µM 149 FAD, 3 µM FMN and 157 pM [14C]L-arginine. The reaction was arrested by the addition (0.5 ml) of a 1:1 suspension of Dowex (AG 50W-8) in water. The mixture was 152 dispersed and diluted with 0.85 ml of distilled water, and 153 allowed to settle for 30 min. The supernatant was removed 154 for estimation of the radiolabelled products by scintillation 155 counting (2 ml Pico-Fluor). Sample protein content was 156 estimated via spectrophotometric assay (Bio-Rad Protein 157 Assay), and NOS activity was expressed as pmol min-1 mg<sup>-1</sup> protein.

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Total NOS activity was defined as citrulline formation 160 that was abolished by incubation in vitro with N<sup>G</sup>-mono- 161 methyl-L-arginine (L-NMMA, 700 μM). The L-NMMA- 162 sensitive NOS activity that was not inhibited by EGTA (1 163 mM) incubation was taken as iNOS activity (László et al., 1995).

#### 2.4. Effect of selective inhibition of inducible nitric oxide 167 synthase on gastric and duodenal albumin leakage

In a separate experiment, the selective iNOS inhibitor, 170 N-(8-(aminomethyl)benzyl)-acetamidine (1400W; 0.2-1 171 mg kg<sup>-1</sup>, s.c.) was administered concurrently with H. 172 pylori lipopolysaccharide. The dose, timing and route of 173 administration of 1400W have been established in previous 174 studies (Garvey et al., 1997; Lamarque et al., 2000). The 175 gastric antrum and the duodenum were removed 4 h 176 following treatment.

#### 2.5. Chemicals

Purified H. pylori lipopolysaccharide was prepared as 181 previously described (Nielsen et al., 1994; Moran, 1999). 182 1400W (N-(8-(aminomethyl)benzyl)-acetamidine) was ob- 183 tained from GlaxoWellcome (Stevenage, UK). L-[U- 184 <sup>14</sup>C]arginine monohydrochloride was obtained from Amer- 185 sham International (UK). [125 I]Human serum albumin was 186 purchased from IZINTA (Budapest, Hungary). All other 187 compounds were from Sigma.

#### 2.6. Statistics

The data are expressed as mean  $\pm$  S.E.M. from n rats 192 per experimental group. For statistical comparisons, the 193 Mann-Whitney non-parallel U-test and the One-way Anal- 194 ysis of Variance followed by the Tukey-Kramer Multiple 195 Comparisons Test were utilised, where appropriate. P < 1960.05 was taken as a significant difference.

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#### 3. Results 199

200 3.1. Induction of nitric oxide synthase and albumin leak-201 202

Administration of purified H. pylori lipopolysaccharide (3 mg kg<sup>-1</sup>, i.v.) led to the expression of iNOS activity in the whole tissue of the gastric antrum (Fig. 1) and duodenum (Fig. 2) when determined 4 h later. The activity of iNOS following challenge was significantly higher in the duodenum than in the antrum (144  $\pm$  58 and 9  $\pm$  5 pmol  $min^{-1} mg^{-1}$  protein, respectively; n = 4, P < 0.05).

The albumin accumulation of non-challenged control rats was  $217 \pm 11$  and  $383 \pm 10 \mu l g^{-1}$  dry tissue in the gastric antrum and duodenum, respectively (n = 6). In a separate group of animals, a significant increase in gastric antral and duodenal vascular albumin leakage occurred 4 h after the injection of purified H. pylori lipopolysaccharide (3 mg kg<sup>-1</sup>, i.v.) as shown in Figs. 1 and 2, respectively. The enhancement of albumin leakage after lipopolysaccharide challenge was significantly greater in the duodenum compared to the gastric antrum ( $\Delta$  322  $\pm$  27 and  $\Delta$  201  $\pm$ 22  $\mu$ l g<sup>-1</sup> dry tissue, respectively; n = 6, P < 0.01).

Macroscopically visible gross damage did not develop either in the gastric antrum or duodenum, over the 4 h

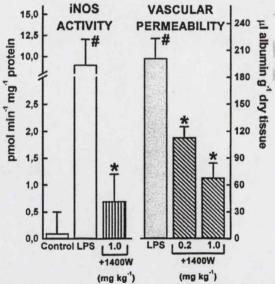


Fig. 1. Increase in inducible nitric oxide synthase enzyme activity (iNOS; pmol min-1 mg-1 protein; left panel) and in vascular leakage (µl albumin g<sup>-1</sup> dry tissue; right panel) in the rat gastric antrum provoked by the intravenous administration of a purified endotoxin (LPS) of H. pylori mg kg-1), determined 4 h later. The effects of the concurrent (3 230 administration of the selective iNOS inhibitor, 1400 W (1 mg kg<sup>-1</sup> and 231 0.2-1 mg kg<sup>-1</sup>, respectively; s.c.) 4 h later are also shown. Data are expressed as mean  $\pm$  S.E.M., where n = 4-6 rats were in a group, where  $^{\#}P < 0.05$  is a significant increase in iNOS activity and albumin leakage 234 following lipopolysaccharide administration; \*P < 0.05 is a significant reduction of lipopolysaccharide-provoked iNOS expression and vascular permeability.

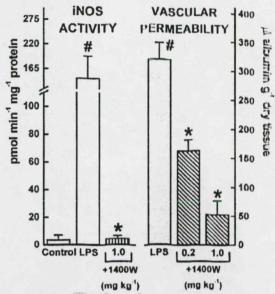


Fig. 2. Increase in inducible nitric oxide synthase enzyme activity (iNOS; pmol min-1 mg-1 protein; left panel) and in vascular leakage (µ1 albumin g<sup>-1</sup> dry tissue; right panel) in the rat duodenum provoked by the intravenous administration of a purified endotoxin (LPS) of H. pylori (3 mg kg<sup>-1</sup>), determined 4 h later. The effects of the concurrent administration of the selective iNOS inhibitor, 1400W (1 mg kg<sup>-1</sup> and 0.2-1 mg 243 kg-1, respectively; s.c.) 4 h later are also shown. Data are expressed as mean  $\pm$  S.E.M., where n = 4-6 rats were in a group, where \*P < 0.05 is a significant increase in iNOS activity and albumin leakage following 246 lipopolysaccharide administration; \*P < 0.05 is a significant reduction of lipopolysaccharide-provoked iNOS expression and vascular permeability.

following lipopolysaccharide administration, nor was it observed in any other region of the gastrointestinal tract.

3.2. Effect of selective inhibition of inducible nitric oxide synthase on gastric and duodenal albumin leakage

Administration of the selective iNOS inhibitor, 1400W (0.2-1 mg kg<sup>-1</sup>, s.c.) concurrently with H. pylori lipopolysaccharide (3 mg kg<sup>-1</sup>, i.v.) caused a significant and 257 dose-dependent reduction of lipopolysaccharide-provoked 258 vascular albumin leakage in the gastric antrum and duodenum determined after 4 h (Figs. 1 and 2). Thus, 1400W (1 mg kg<sup>-1</sup>, s.c.) reduced albumin leakage in the duodenum and in the gastric antrum by  $86 \pm 4\%$  and  $68 \pm 6\%$ , respectively (P < 0.05; n = 4).

Likewise, 1400W (1 mg kg<sup>-1</sup>, s.c.) caused a  $92 \pm 6\%$ (n = 4, P < 0.05) and  $98 \pm 1\%$  (n = 4, P < 0.05) inhibition of iNOS activity, determined 4 h after challenge, in 266 the gastric antrum and duodenum, respectively (Figs. 1 and 267

#### 4. Discussion

271 In the present in vivo study, an increase in albumin leakage, a sensitive index of vascular cell injury and

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inflammation, in the rat gastric antrum and duodenum 275 could be observed 4 h following the intravenous administration of H. pylori lipopolysaccharide. These findings of gastroduodenal microvascular dysfunction agree with previous observations, where increases in microvascular permeability to protein in the rat mesenteric circulation following local application of a water-extract of H. pylori have been observed (Kurose et al., 1994). In addition, administration of H. pylori extract or purified lipopolysaccharide has been shown to provoke injury of duodenal epithelial cells following intravenous challenge (Lamarque et al., 1998; Lamarque et al., 2000). 285

The mechanisms by which soluble mediators derived from H. pylori can injure the gastroduodenal microvasculature could involve a number of pathways. Early studies suggested that H. pylori extract-induced microcirculatory albumin leakage could be the consequence of interstitial and intravascular cell-cell interactions (Kurose et al., 1994). Thus, the lipopolysaccharide from H. pylori can activate polymorphonuclear cells (Nielsen et al., 1994). Administration of H. pylori extract can lead to the expression of a neutrophil-activating protein, which causes neutrophils to adhere to the vascular endothelium, an event that can initiate microcirculatory protein leakage (Evans et al., 1995; Takemura et al., 1996). Neutrophils are known to release a number of pro-inflammatory mediators, includ-300 ing platelet-activating factor and thromboxanes, which have an important role in promoting vascular albumin leakage following lipopolysaccharide challenge (László et al., 1994) as well as producing cytotoxic radicals such as superoxide and NO (Moncada and Higgs, 1995).

304 In the present study, the involvement of iNOS in the processes leading to microvascular injury following challenge with H. pylori lipopolysaccharide was evaluated by using a highly selective iNOS inhibitor, 1400W (Garvey et 309 al., 1997; László and Whittle, 1997). Thus, in doses that 310 reduced the iNOS enzyme activity following lipopoly-311 saccharide challenge, 1400W substantially attenuated the 312 albumin leakage in both gastric antrum and duodenum. 313 These findings thus extend to the microvasculature, the 314 previous observations that 1400W can prevent injury to 315 duodenal epithelial cells provoked by H. pylori lipopoly-316 saccharide (Lamarque et al., 2000). Such studies thus provide good evidence that iNOS is involved in both the 318 epithelial cell cytoxicity and the microcirculatory injury 319 brought about by systemic exposure to H. pylori lipopoly-320 saccharide, a process that may involve the production of peroxynitrite through the combination of superoxide and 322 NO (Lamarque et al., 2000). Despite substantially greater 323 iNOS activity being detected in the duodenum compared 324 to the gastric antrum, the relative expression of iNOS in 325 these tissues appeared to contribute significantly to the 326 microvascular injury, as 1400W prevented the leakage in 327 both regions. The sites of iNOS expression that promote 328 vascular leakage in the present model are not known but

may involve the microvascular endothelium. In clinical

studies on gastric mucosal biopsies from patients with 330 gastritis associated with H. pylori infection, increased antral mRNA for iNOS, and iNOS protein in epithelium, 332 endothelium and inflammatory cells have been reported 333 (Fu et al., 1999).

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The present results thus suggest that the purified lipo- 335 polysaccharide from H. pylori is capable of inducing an 336 iNOS-mediated microvascular inflammatory response in 337 the gastric antrum and duodenum of the rat following its parenteral administration. Thus, the local liberation of H. 339 pylori lipopolysaccharide into the mucosal tissue, or into 340 the systemic circulation, may contribute to the pathogenic 341 processes provoked by H. pylori, through the expression 342 of iNOS.

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### Vasopressin pressor receptor-mediated activation of HPA axis by acute ethanol stress in rats

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László, Ferenc A., Csaba Varga, Imre Pávó, János Gardi, Miklós Vecsernyés, Márta Gálfi, Éva Morschl, Ferenc László, and Gábor B. Makara. Vasopressin pressor receptor-mediated activation of HPA axis by acute ethanol stress in rats. Am J Physiol Regulatory Integrative Comp Physiol 280: R458-R465, 2001.—The plasma arginine vasopressin (AVP), ACTH, and corticosterone levels and the hypothalamic corticotropin-releasing hormone (CRH) content were measured after oral administration of 1 ml of 75% ethanol to rats, a model known to induce acute gastric erosions and stress. Elevated plasma AVP, ACTH, and corticosterone levels were detected 1 h after ethanol administration. Treatment with the vasopressin pressor (V<sub>1</sub>) receptor antagonist [d(CH2)6Tyr(Me)-AVP] before ethanol administration significantly reduced the ACTH and corticosterone level increases. A higher hypothalamic CRH content was measured at 30 or 60 min after ethanol administration. V1 receptor antagonist injection, 5 min before ethanol administration, inhibited the rise in hypothalamic CRH content. The protein synthesis blocker cycloheximide prevented the hypothalamic CRH content elevation after stress. The AVP-, CRH-, and AVP + CRH-induced in vitro ACTH release in normal anterior pituitary tissue cultures was also prevented by pretreatment with the V<sub>1</sub> receptor antagonist. The results support the hypothesis that stress-induced AVP may not only act directly on the ACTH producing anterior pituitary cells but also indirectly at the hypothalamic level via the synthesis and release of CRH.

adrenocorticotropic hormone; corticosterone; hypothalamic corticotropin-releasing hormone;  $V_1$  receptor antagonist

A HIGH DOSE OF ORALLY ADMINISTERED ethanol generates gastric erosions in rats (61), and such lesions are often used to evaluate the gastric action of various drugs. Similar lesions can be observed in the gastric mucosa in animals exposed to different stress situations such as cold restraint, endotoxin injection, or hemorrhagic stress (28). Adrenal medullectomy (66), endogenous vasopressin [arginine vasopressin (AVP)] deficiency (29), administration of a pressor (V<sub>1</sub>) AVP antagonist (29), and orchidectomy (27) all protect the gastric mu-

cosa against ethanol-induced damage. In contrast, adrenalectomy attenuates gastric mucosal protection against ethanol injury (66). These data demonstrate that various hormones participate as facilitatory or inhibitory mediators in the ethanol-induced generation of acute gastric nucesal stress crossions.

Among the stress hormones, ACTH is one of those responding most sonsitively to various stimuli, and it is therefore a useful indicator for the monitoring of stress situations. The major physiological regulator of ACTH secretion following stress is corticotropin-releasing hormone (CRH), originating from the hypothalamus (21).

The aims of the present study were to learn more about hormonal changes following orally administered high-dose ethanol (known to cause gastric erosions) and the involvement, if any, of AVP in the changes observed following acute ethanol challenge.

#### MATERIALS AND METIIODS

Animals. Male Wister rats (from our breeding farm originating from different litters) weighing 180–220 g were fasted for 24 h, but they received water ad libitum. The animal care and research protocols were in accordance with the guidelines of our university. The animal house had artificial lighting (from 6 AM to 6 PM). Five rats were kept per cage. The rats had been handled and treated orally three times daily (8 AM, 2 PM, and 8 PM) with 1 ml of tap water via a gastric tube for 6 consecutive days before the experiment. This procedure was designed to accustom the rats to the presence of humans and to the insertion of the gastric tube to minimize the effects of nonspecific stress.

Measurements of plasma AVP, ACTH, and corticosterone levels in response to ethanol administration in the absence or presence of a V<sub>1</sub> antagonist. Each experimental group consisted of 10 rats. In 10 groups, 1 ml of 75% ethanol or 1 ml of tap water (as control) was administered orally to the animals via a gastric tube. Zero, 5, 15, 30, or 60 min after ethanol or water administration, the rats were killed by decapitation between 8 and 9 AM, and trunk blood was collected in polystyrene tubes containing 180 µl of 1.6 M EDTA. Blood samples were kept on ice and centrifuged (3,000 rpm) at 4°C

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to obtain plasma for AVP, ACTH, and corticosterone determinations. A further 10 groups were treated with a highly selective  $V_1$  AVP receptor antagonist 5 min before ethanol or water administration. The  $V_1$  receptor antagonist [1-( $\beta$ -mercapto- $\beta$ , $\beta$ -cyclopentamethylenepropionic acid)-2-(O-methyl)-Tyr, Arg8|VP, called Manning peptide and abbreviated as d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP, was used at a dose of 1.0  $\mu$ g/kg body wt ip (41). The receptor-antagonist dose was determined according to earlier published data (28, 31). The animals were killed at the above different times after ethanol or water administration, and their plasma was collected similarly.

Plasma AVP level. Plasma AVP level was determined with a specific RIA system (25). The extraction was performed on RPN 1902-C8 minicolumns (Amprep, Amersham, UK), with a recovery of >95%. Synthetic AVP (Arg<sup>8</sup>-vasopressin; Organon, Oss, The Netherlands) was used for antibody production, for the preparation of the tracer, and as a standard. The cross-reactivities of the AVP antibody used in the RIA were 0.03% with vasotocin, <0.01% with oxytocin, and 0.03% with ACTH<sub>1-24</sub>. The sensitivity of the RIA was 1 pg/ml. The intraand interassay coefficients of variation proved to be 13.3 and 16.3%, respectively.

Plasma ACTH level. A direct RIA method was used (24). Rabbits were immunized with ACTH<sub>1-32</sub> to obtain an antibody. The sensitivity of the method was 1 pg/ml, and the intra- and interassay coefficients were 4.7 and 7%, respectively. The cross-reaction with  $\alpha$ -melanotrophic hormone was <0.2%.

Plasma corticosterone level. This method was described by Mihály et al. (45). Twenty-one-corticosterone-albumin conjugate was used for immunization in rabbits. Endogenous corticosteroid-binding globulin was inactivated with methanol. A direct RIA was used. The sensitivity of the method was 3  $\mu$ g/dl, and the intra- and interassay coefficients were 9 and 13.7%, respectively. The cross-reactions with progesterone and estrone were 7.3 and 0.1%, respectively.

Measurements of CRH content of hypothalamus. After the rats' decapitation, brains were quickly removed, and the hypothalamus was isolated on ice. The hypothalamus was defined as the tissue within 3 mm of the ventral surface of the brain, within the following borders: optic chiasm, mamillary bodies, and lateral hypothalamic sulci. The whole stalkmedian eminence was included in the hypothalamic preparations. CRH concentration was determined by a RIA technique (64). The hypothalamus was homogenized with ultrasound (Soniprep 150 MSE) in 100 mM HCl containing 1 mM ascorbic acid, and an aliquot was taken for protein measurements (38). The residual homogenate was centrifuged at 6,000 g for 20 min at 4°C, and aliquots were taken and lyophilized for RIA. The CRH antiserum (P. Vecsei, Heidelberg, Germany) was obtained from a rabbit immunized with human CRH. The CRH antibody (43) was specific for the C-terminal region of the  $CRH_{1-41}$  molecule because it did not cross-react with the fragments  $CRH_{1-20}$  and  $CRH_{6-}$ 33. The CRH tracer was prepared with the use of a modified iodogen method to minimize damage to the iodinated peptide (35). The labeled material was purified via two steps of reverse-phase chromatography (17), a gradient HPLC system being applied in the second step. The specific radioactivity of the purified tracer was 1,700-1,900 Ci/mmol. The freezedried residues were redissolved in 1 ml of assay buffer [50] mM phosphate (Sigma), pH 7.4, containing 0.25% human serum albumin (Izinta) and 0.1% Triton X-100 (Reanal)], and 200-µI aliquots were subjected to RIA. The RIA standard was a synthetic human/rat (h/r) CRH preparation (Bachem, Budendorf, Switzerland).

The procedure involved a nonequilibrium system; a 16-h preincubation of the samples or standards with antiscrum (100 µl, working dilution 1:10,000 was followed by a 24-h incubation with corticotrophic-releasing factor tracer (100 µl, 10,000 cpm). The immunologically bound and free fractions were separated with a second antibody (raised in our laboratory in sheep against whole rabbil lgt4) and subsequently by polyethylene glycol (PEG) 6,400 precipitation (Ferak Laboratory, Berlin, Germany) by the double-antibody/PEG method. The lower limit of assay detection was 7-8 pg/tube. The intra- and interassay conflictents of variation were 4.0 and 13.8%, respectively. CHH immunoreactivity in hypothalamus extracts subjected to HPLC has been shown to cochromatograph with synthetic laft (RH<sub>1-4</sub>) (64). The CRH content of the hypothalamus is expressed in pirograms per milligrams protein. Each group consisted of 10 rats.

To determine whether the hypothalamic CRH response to ethanol stress can be necribed to the increased protein synthesis, rats were treated with the protein synthesis-blocking compound cycloheximide before oral ethanol administration. Thirty minutes before ethanol administration, an injection of 0.9% NaCl (Sigma) or of cycloheximide (10 or 30 mg/kg body wt; Fluka, Buchs, Switzerland) dissolved in 0.9% NaCl was administered intraperitoneally. The rate were killed 30 or 60 min after ethanol administration, and the CRH content of the hypothalamus was measured.

Anterior pituitary tissue culture. Manolayer pituitary cell cultures of Wistar rats weighing 180-230 g wore prepared (19). Ten pituitaries were used in every tissue culture experiment. The hypophysis was sterile-removed immediately after decapitation of the rats. The unterlar lobe of the pituitary gland was isolated under a preparative microscope, and the tissue was digested in the presence of trypsin, collagenase, deoxyribonuclease I and II, and dispase ((IIBCO). The dispersed cells were placed in plustic collagen-conted Petri dishes and suspended in Dulbeago's modified Eagle's medium supplemented with 20% fetal having serum (GIBCO). The cultures were maintained at 37°(1 in a humidified atmosphere of 10% CO2 in air and washed every 3 days. Experiments were performed on 14-day cultures. We performed the standardization of the cell cultures by immunoreactivity of ACTH at the start and at the end of experiments, determining the relative frequency of immunoreactive ACTH containing cells per unit area. If the relative frequency proved to be no more than 5 to 7%, the cell cultures were used. The standardized monolayer cell cultures were functionally controlled with potassium (30 mM, 30 min) as a nonspecific releasing agent. Viability was 97-100%. In our experimental conditions, the stable equilibrum in ACTH release developed after ~90 min. This was the reason for the use of the longer incubation period. The viability did not change during incubation. During pilot experiments, we tried several doses of AVP, CRH, and V<sub>1</sub> antagonist, and we used the maximal effective dose (10<sup>-6</sup> M) later. The colls were incubated for 3 h with 10<sup>-6</sup> M AVP or 10<sup>-6</sup> M CRH (Bachem) alone or 10<sup>-6</sup> M AVP + 10 "6 M CRH together, for 3 h with 10 "6 M V, receptor antagonist alone, and for 2 h with 10.00 M V, receptor antagonist followed by 1 h with 10.00 M AVP or 10.00 M CRH alone or 10.00 M AVP + 10.00 M CRH tagether. During the control period, the incubation was performed without any additions. The ACTH concentrations from 100-11 supernatant media samples were measured by direct RIA (24). The method of ACTH determination was the same as in the case of plasma. The data were calculated on the basin of the 12 measurements in each group.

Statistics. All data are presented as means ± SE. The Mann-Whitney's U test was used for comparisons involving

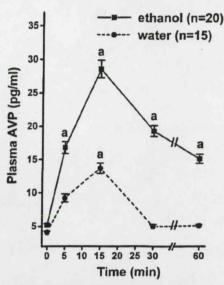


Fig. 1. Effect of 75% ethanol administered orally (1 ml/rat) on the plasma arginine vasopressin (AVP) level. Vertical bars denote means  $\pm$  SE;  $^{a}P < 0.05$  vs. control group.

two groups. The curves were analyzed via the Kruskal-Wallis test. A probability level of <0.05 was accepted as indicating a statistically significant difference.

#### RESULTS

Plasma AVP level. The oral administration of water led to slightly increased plasma AVP levels at 5 and 15 min, but the AVP level later returned to the normal range. Elevated AVP levels were detected at 5, 15, 30, and 60 min after ethanol administration. The maximum AVP concentration was measured at 15 min after

ethanol (Fig. 1).

Plasma ACTH level. The baseline plasma ACTH and corticosterone levels are relatively high and probably reflect the cumulative effects of fasting and the response to the control procedure. Water similarly led to increased plasma ACTH levels at 5 and 15 min, but normalization had occurred by 30 min. V1 receptor antagonist injection before water administration prevented the transient increase in ACTH concentration. Ethanol administration resulted in increased ACTH levels between 5 and 60 min. The ACTH levels decreased 15 min after every treatment, possibly because of the feedback action of the increased corticosterone level. Treatment with the V<sub>1</sub> receptor antagonist before ethanol administration significantly inhibited the ACTH rise normally seen between 15 and 60 min, but the normal range was not reached (Fig. 2).

Plasma corticosterone level. The plasma corticosterone levels were also higher between 5 and 15 min after water administration. V<sub>1</sub> receptor antagonist injection significantly decreased the plasma corticosterone level between 5 and 10 min after water administration. Ethanol yielded an elevated plasma corticosterone level at 5 min, and the level remained high up to 60 min. V<sub>1</sub> receptor antagonist injection reduced the plasma corticosterone concentrations be-

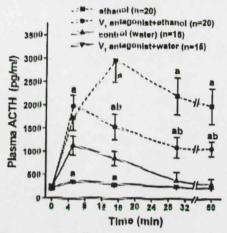


Fig. 2. Effect of pressur  $(V_1)$  receptor antagonist on the plasma ACTH level after 75% ethanol administration (1 ml/rat). Vertical bars denote means  $\pm$  SE;  $^{n}P \leq 0.05$  vs. control group,  $^{n}P < 0.05$  vs. group treated with ethanol alone.

tween 15 and 60 min after ethanol administration (Fig. 3).

Hypothalamic CRH content. As Fig. 4 shows, water administration through the gastric tube did not induce any change in the CRH content of the hypothalamus. Similar results were observed after V<sub>1</sub> receptor antagonist and water administration. Higher contents of hypothalamic CRH were measured at 30 and 60 min after ethanol administration. The V<sub>1</sub> receptor antagonist injected 5 min before ethanol administration blocked the rise in hypothalamic CRH content, which then remained within the control range after ethanol stress.

Actions of cycloheximide on CRH. After cycloheximide and oral water administration, there was no change in the hypothalamic CRH content (Fig. 5).

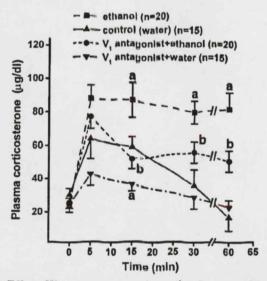


Fig. 3. Effect of V<sub>1</sub> receptor antagonist on the plasma corticosterone level after 75% ethanol administration (1 ml/rat). Vertical bars denote means  $\pm$  SE;  $^{o}P < 0.05$  vs. control group,  $^{b}P < 0.05$  vs. group treated with ethanol alone,

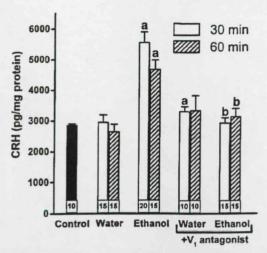


Fig. 4. Effect of  $V_1$  receptor antagonist on the hypothalamic corticotropin-releasing hormone (CRH) content after 75% ethanol administration (1 ml/rat). Vertical bars denote means  $\pm$  SE;  ${}^{\rm o}P < 0.05$  vs. control group,  ${}^{\rm b}P < 0.05$  vs. corresponding group treated with ethanol alone.

Higher CRH levels were detected after ethanol administration. Smaller doses of cycloheximide treatment before ethanol administration significantly inhibited the hypothalamic CRH enhancement; however, the CRH concentration remained above the control level at both 30 and 60 min after ethanol administration. However, higher doses (30 mg/kg body wt) of cycloheximide prevented the increase in the hypothalamic CRH content after ethanol administration. Inhibition of protein synthesis with cycloheximide dose dependently reduced the stress-induced increase in CRH in the hypothalamus, as shown in Fig. 5.

ACTH levels in pituitary tissue culture. Increased ACTH levels were demonstrated in the anterior pituitary tissue culture media after the addition of 30 mM

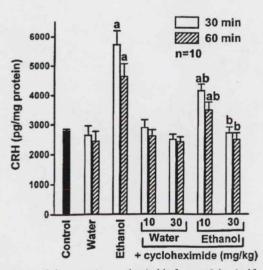


Fig. 5. Effect of the protein synthesis blocker cycloheximide on the hypothalamic CRH content after 75% ethanol administration (1 ml/rat). Vertical bars denote means  $\pm$  SE;  $^{\alpha}P < 0.05$  vs. control group,  $^{b}P < 0.05$  vs. corresponding group treated with ethanol alone.

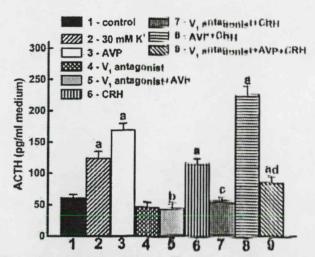


Fig. 6. Effects of AVP, CRH, and  $V_1$  receiter antagonist on the ACTH secretion in vitro in the anterior pituitary tissue culture. Vertical bars denote means  $\pm$  SE, where n=12 in every group. "P<0.05 vs. control group,  $^bP<0.05$  vs. group treated with AVP alone, "P<0.05 vs. group treated with CRH alone, and "P<0.05 vs. group treated with AVP + CRH.

K<sup>+</sup> to the incubation medium, due to a depolarization-induced ACTH release (Fig. 6). The ACTH concentration was significantly enhanced after the administration of 10<sup>-6</sup> M AVP or 10<sup>-6</sup> M CRH alone. A higher ACTH level was found after the administration of AVP and CRH together. A slightly decreased ACTH release was observed after V<sub>1</sub> receptor antagonist administration alone. The AVP- or CRH-induced, increased release of ACTH was prevented by the administration of V<sub>1</sub> receptor antagonist. A moderate enhancement of ACTH level was detected when AVI + CRH was administered after the V<sub>1</sub> receptor antagonist treatment. The data were calculated on the basis of 12 measurements in each group.

#### DISCUSSION

The series of studies presented here shows that the plasma AVP, ACTH, and corticosterone levels and the hypothalamic CRH content are elevated after acute stress induced by a high concentration of orally administered ethanol, and these responses can be reversed by an AVP V1 receptor antagonist. These findings are in agreement with previous observations that a high dose of orally administered ethanol causes AVP release in humans and in rats (27, 34). These results, however, are in apparent conflict with the established finding that ethanol in low concentration inhibits the release of AVP (54, 63). The paradoxical action of orally administered ethanol on AVP release might possibly reflect the high dose of ethanol used. It is known that after stress, CRH and AVP are coreleased into the primary capillaries of the portal circulation in the stalk-median eminence region from where the blood carries the neuropeptides to the anterior lobe. This AVP is unlikely to reach the systemic circulation in quantities high enough to elevate the plasma AVP level. Instead, the plasma AVP changes probably reflect the activity of the

magnocellular neurons with terminals in the neural lobe, and this AVP may reach the anterior lobe only after dilution in the general circulation (21). We have observed that the AVP elevation is sufficiently high to influence the hypothalamic CRH cells after administration of a high dose of ethanol into the stomach, as shown by the preventive effect of the V<sub>1</sub> receptor antagonist. We consider it likely that stimulation of the CRH/AVP containing parvocellular neurons will be involved in the release of ACTH and corticosterone after ethanol administration.

Our experiments revealed increased plasma AVP and hypothalamic CRH contents after ethanol administration. There was no elevation of the CRH content in the hypothalamus after ethanol administration when the V<sub>1</sub> receptor antagonist was injected before ethanol administration. This suggests that systemic AVP sends signals via the  $V_1$  receptors to the hypothalamus to stimulate either directly or indirectly the CRHproducing hypothalamic neurons. We suggest that AVP may act in a dual manner: 1) in the pituitary portal blood, AVP and CRH are cosecreted, and AVP potentiates the actions of CRH; and 2) in the systemic circulation, AVP may signal the presence of stress to the hypothalamus (21). Many data in the literature provide evidence of a physiological synergistic interaction between CRH and AVP at the level of the pituitary. Exogenously administered AVP significantly potentiates CRH-stimulated ACTH release in rats (12, 67) and humans (26, 37). Endogenous AVP can also potentiate CRH-stimulated ACTH secretion in humans; synthetic human CRH resulted in higher ACTH and cortisol responses after a water restriction than after a water load (69). The role of endogenous AVP in the ACTH response to stress is suggested by the results of Guillaume et al. (13), who found significantly reduced ACTH and cortisol responses to insulin stress and restraint stress and CRH injection in anti-AVPimmunized rams compared with controls. In accordance with our finding, Rivier et al. (58) reported that ACTH secretion induced by ether stress, which stimulates CRH secretion, is significantly moderated by pretreatment with an AVP antagonist analog. All of these data are compatible with a dual role of AVP in the regulation of the hypothalamic-pituitary-adrenocortical (HPA) axis.

The increased plasma ACTH and corticosterone levels in response to a high dose of orally administered ethanol relate to a stress situation (55-57). During stress, AVP plays an important role in ACTH stimulation. The involvement of AVP in the ACTH response to restraint stress is supported by the observation that the ACTH response is impaired in genetically AVPdeficient Brattleboro rats (5, 11, 22, 44, 70, 71). Furthermore, the ACTH responses to ether stress and adrenalectomy are blunted in Brattleboro homozygous rats (11). As Brattleboro rats do not differ from the normal controls with respect to the hypothalamic CRH content (22), the impaired ACTH response in these rats is most probably explained by their AVP deficiency. The role of endogenous AVP in stress-induced ACTH release han previously been demonstrated in several studies involving the use of the passive immunization technique with an AVI' antiserum or the administration of synthetic AVP antagonists. In the rat, intraperitoneal administration of anti-AVP immunoglobulins reduces the ACTII responses to rostraint stress and formalin stress (96). The intracerebroventricular administration of AVP antiserum leads to a moderate but significant reduction in plasma ACTH level after ether stress (50). V, receptor antagonists attenuate the AVPinduced increase in plasma ACTH when administered before AVP injection in the rat (4, 18). Besides the animal observations, we have some human experiences (32), i.e., the incidence of human gastroduodenal ulceration is significantly higher in the normal population (in whom the release of AVP is presumed to be intact) than in the AVP-deficient population (central diabetes insipidus patients). These findings indicate that endogenous AVP plays an aggressive role in the development

of gastrointestinal mucosal injury.

The question arises of how ACTH secretion is influenced by AVP after stross. We refer here to the minireview by Kjaer (21); the action of AVP on ACTH release is exerted in direct and indirect ways. The direct effect influences the ACTH production of the anterior pituitary gland. The principal effect of systemic AVP on ACTH secretion in conscious rats is produced indirectly via the stimulation of hypothalamic CRH. The indirect effect of AVP is probably more significant when AVP is administered in vivo (47), because it is known to have marked cardiovascular effects even in low concentrations. Moreover, the pituitary AVP receptor differs from the classical V1 receptor, and the designation  $V_{1b}$  (as opposed to  $V_{1a}$  for the classical receptor) has been proposed (4, 6, 22, 23). Under in vivo conditions, however,  $V_{1a}$  receptors appear to be involved, because  $V_{1a}$  receptor antagonists were effective in inhibiting the release of ACTH or corticosterone (2, 9, 40, 42, 47, 59, 60, 65) and because the pressor activity of V<sub>1</sub> receptor agonists correlated with their ability to release ACTH and corticosterone (2, 47). Our in vivo results fit with the above explanation; the ACTH and corticosterone secretions after ethanol stress were considerably inhibited if the rats wore treated with the V<sub>1</sub> receptor antagonist d(CH<sub>2</sub>), Tyr(Me)AVP immediately before ethanol administration. Our in vitro experiments, however, indicate that the ACTH-blocking effect of the V<sub>1</sub> receptor antagonist does not develop only through the decrease of the hypothalamic CRH content; in high concentrations, the compound can also directly block the ACTH-increasing effect of AVP in the anterior tissue culture. The observation with AVP demonstrates that d(CH<sub>2</sub>)<sub>5</sub>Tyr(Me)AVP is a mixed antagonist with direct V11, receptor antagonist character, which is in accordance with the findings of Jard et al. (18), Antoni et al. (4), and Bernardini et al. (8). The latter finding with CRH is rather surprising, and it might possibly relate to a still unidentified interaction of V<sub>1</sub> receptor antagonists with CRH receptors on the pituitary ACTH-producing cell;

however, this phenomenon needs further experimental validation.

For clarification of the role of CRH in the stress reaction after ethanol administration, the alterations in hypothalamic CRH content were also determined after high doses of orally administered ethanol. Many researchers have investigated the effects of different types of stress on the CRH content in the hypothalamus (51). Although determination of the CRH content in the hypothalamus alone is insufficient to establish whether the changes in release, storage, or synthesis are responsible, a difference between treated and control groups clearly reflects the involvement of CRH in the stress situation. Chappell et al. (10) reported that acute or chronic stress resulted in a 50% decrease in hypothalamic CRH content. Similar observations were published following acute (49, 52) or chronic stress (3). These reductions are thought to relate to the secretion of CRH from the hypothalamus in the acute situation, and to continued release in the chronic stress condition, when new CRH synthesis cannot keep pace with the demands for higher secretion. This hypothesis, however, has not yet been proved convincingly (51). Murakami et al. (48) reported a fast increase in the hypothalamic CRH content, already observed 2.5 min after ether stress. Moldow et al. (46) described a significant decrease in hypothalamic CRH content 15 min after the initiation of restraint stress. This was followed by an enhanced hypothalamic CRH concentration at 60 min, which could be blocked by cycloheximide administration before stress, indicating that new protein synthesis can explain the increased hypothalamic CRH level demonstrated at this time point. Analogous results were reported by Haas and George (14); a significant increase in hypothalamic CRH concentration was observed 24 h after a single 5-min foot shock, and when protein synthesis was eliminated by anisomycin pretreatment, it completely abolished the increase in hypothalamic CRH content. A number of recent investigations has directly studied CRH gene expression by measuring hypothalamic CRH mRNA concentrations. Different stressors, which activate the hypothalamic-adrenal axis, can also influence CRH gene expression. Swimming, hypertonic saline, or restraint stress led to an increased hypothalamic CRH mRNA expression within 4 h, and the level remained elevated for 24 h (15, 16, 33). Similar findings were observed after a 2-h immobilization stress (39) and a 1-h restraint stress (20). In our experiments, the increases in hypothalamic CRH content were blocked by cycloheximide pretreatment, which points to a role of enhanced protein synthesis in the increased hypothalamic CRH concentration after ethanol stress (1, 53). Whether the overall increase in hypothalamic CRH content, paralleling an enhanced release in our studies, reflects a rapid increase in translation and/or increased processing of the prohormone of CRH requires further studies.

In their review, Owens and Nemeroff (51) presented cumulative evidence that CRH integrates the overall physiological behavioral responses of an organism to stress. The neuroendocrine response to stress is primarily controlled by CRII neurons originating from the paraventricular nucleus of the hypothalamus, but the AVPergic magnocellular neurons breammably also participate in the development of a stress reaction. Our present results support the importance of the hypothalamo-neurohypophyseal or magnocellular AVP system in the development of physiological or pathological changes following various stress situations. It also suggests possible pharmacological strategies involving AVP antagonist agents to prevent such pathology.

#### Perspectives

Earlier we studied the development of gastrointestinal stress erosions in different rat experimental models, including mucosal injury provoked by otherol, indomethecin, cold-restraint stress, and hemorrhagic and endotoxin shock (26, 68). We found that an increased AVP secretion during these stress situations has key importance in the initiation of damage (31). In the present work, the interaction between the HPA axis and AVP secretion was demonstrated following acute ethanol challenge. The lower incidence of gastrointestinal ulceration among AVP-deficient patients with central diabetes insipidus gives further support to the pathological importance of endogenous AVP release in the development of mucosal damage even under clinical circumstances (32).

Gastrointestinal mucosal stress erosions commonly appear among critically ill patients mostly in intensive care units after a number of acute conditions, such as severe trauma, burns, septic or homorrhagic or cardiogenic shock, or injury of the central nervous system, etc. (62). Once hemorrhage becomes manifest, the severity of the condition is reflected in the mortality rate, which approaches 50% (62). Although it would be of high clinical significance, the problem of how to prevent the development of auch niucosal erosions is not solved (7). The findings in earlier studies suggested that the use of vasopressin pressor recoptor (V1) antagonists in clinical practice might have potential therapeutic benefit (28, 29, 31, 32). The present results revealed that a vasopressin pressor receptor (V1) antagonist could not only prevent the vasoconstrictive action of increased vasopressin secretion, but it could also block the elevation of the hypothalamic CRH response and ACTH-corticosterone secretion following acute ethanol challenge. These experimental and clinical observations lead us to conclude that vasopressin antagonists might have importance in the prevention of the generation of the life-threatening bleeding from gastrointestinal stress erosions.

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Endogenous bacteria-triggered inducible nitric oxide synthase-mediated increase of mucus secretion protects the stomach of ovariectomized rat.

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## Endogenous bacteria-triggered inducible nitric oxide synthase activation protects the ovariectomized rat stomach

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#### Abstract

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Under experimental circumstances, ovariectomy attenuates gastric mucosal injury where nitric oxide (NO)-mechated pathways are involved. In our study, we have examined the changes in constitutive (cNOS) and inducible NO synthase (iNOS) cazyme activities (assessed by the citrulline assay), and the role of endogenous bacteria in overiectomy-provoked mucosal defence. Gastric lesions were induced by indomethacin (50 mg/kg, s.c.) over a 4 h period in sham-operated and ovariectomized female Wistar rats. Groups of animals received the wide-spectrum antibiotic ampicillin (800 mg/kg/day, p.o., for 3 days), and others were injected with bacterial endotoxin (E. coli, 3 mg/kg, i.v., 5 h before autopsy). We found that ovariectomy increased iNOS and decreased eNOS activity (resulting an elevated total gastric NO level), and protected the stomach, effects were reversed by ampicillin freatment. In ovary-intact rats, administration of bacterial endotoxin enhanced gastric iNOS activity and reduced lesion-formation. These results suggest that ovariectomy improves gastric mucosal defence by endogenous bacteria-triggered activation of iNOS, an 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Oestrogen; Inducible nitric oxide synthase; Constitutive nitric oxide; Synthase; Gastric mucosal defence; buzyme regulation

#### 1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by three distinct isoenzymes that can be divided into two functional classes based on their sensitivity to calcium. The cytokine- or bacterial endotoxin-inducible isoenzyme (iNOS) is expressed mostly under pathological conditions, and binds calmodulin tightly in a calcium-independent fashion. The constitutive form (cNOS) is expressed continuously under physiological circumstances, and binds calmodulin in a reversible and calcium-dependent fashion. Inhibition of cNOS leads to vasoconstriction, to the aggregation of platelets, to an increase in vascular permeability and adhesion of neutrophils to the vascular endothelium, processes, which are deeply involved in the development of vascular endothelial dysfunction [9,10].

In vitro observations suggested that the liberation of NO from vascular tissues is regulated by sex hormones

<sup>[20],</sup> where oestrogens enhance NO release from vascular tissues [16,20]. In vivo, we recently confirmed the oestrogen-mediated up-regulation of cNOS in the rat heart and aorta by direct measurement of enzyme activity [11]. In addition, extensive evidence supports the theory that sexual steroids are involved in gastrointestinal defensive mechanisms [1,4], where N() also plays a key role in the maintenance of mucosal integrity of the gut [21]. Indeed, 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 [5-7]. Moreover, decreased cNOS activity and mucus level were found in the stomach in water immersion restraint stress-induced ulceration, effects reversed with NO synthase inhibition [14]. In contrast, in the oestrogen delicient rat stomach, we found an enhanced total NO synthase activity and mucus secretion, while the gastric mucosa was less susceptible towards ulcerogenic challenge [12]. This had been a result of the increased NO production, since the pharmacological blockage of NO synthesis abolished ovariectomy-provoked elevation of mucus secretion and mucosal protection [12].

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In this present study, we investigated the changes in the ratio of cNOS and iNOS activity in the stomach of the oestrogen-deficient rat, in conjunction with, a possible role of endogenous bacteria in ovariectomy-provoked mucosal defence.

#### 2. Materials and methods

#### 2.1. Experimental protocol

Female Wistar rats (10-12 weeks-old) were used. Ovariectomy and sham-operation had been performed under ketamine/medetomidine (75 mg/kg; 0.5 mg/kg) narcosis. The animals were allowed to recover over 1 month. Gastric mucosal injury was induced by indomethacin (50 mg/kg, s.c.) over a 4 h period. Before any experimentation, the animals were starved for 24 h, but had free access to water. Following autopsy, the extent of lesions in the stomach was determined by a digital planimeter, the ratio of injured parts was compared with the total mucosal surface, and the data were expressed as a per cent. 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 autopsy, i.e. the latter 2 h before indomethacin challenge. 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.

#### 2.2. Measurement of nitric oxide synthase enzyme activity

NOS activity was determined as the conversion of L-[14C]-arginine monohydrochloride to L-[14C]-citrulline based on the method described previously [17,22] with minor modifications aiming to detect mostly the activity of cNOS [2,3,11,20]. We sacrificed the animals by decapitation, and immediately after autopsy, we prepared the fresh tissues for NOS measurements. Protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), and NOS activity was expressed as pmol/min/mg protein. Total NOS activity was defined as citrulline formation that was abolished by incubation in vitro with NG-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 iNOS activity was also determined, as the difference between samples containing 1mM EGTA and samples containing 1 mM L-NNA.

#### 2.3. Chemicals and statistics

L-[U-14C]-arginine monohydrochloride was obtained from Amersham International. All other compounds

were from the Sigma Chemical Company. Data are expressed as the mean  $\pm$  S.E.M. 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.

#### 3. Results

In the ovariectomized rat stomach, the total NO synthase activity was enhanced compared with the ovary-intact sham-operated females (by  $73\pm9\%$ , n=10; P<0.005). In the gastric tissues of these oestrogen-deficient animals, we found a significant decrease in cNOS activity (by  $31\pm8\%$ ; n=18; P<0.05), and an increase in iNOS activity (by  $719\pm10\%$ ; n=10; P<0.001) as shown in Fig. 1.

Administration of indomethacin induced gastric mucosal lesions (involving  $5\pm0.5\%$  of the total gastric mucosal surface, n=10, P<0.001), which were attenuated by ovariectomy (by  $64\pm4\%$ , n=5, P<0.001). Administration of ampicillin reversed ovariectomy-provoked gastric mucosal defense after indomethacin challenge (by  $94\pm11\%$ , n=5, P<0.05), and abolished the increase in iNOS activity (by  $99\pm1\%$ , n=5, P<0.001) in the ovariectomized rat. Data are shown in Fig. 2. During this study, no change in cNOS activity could be observed (data are not shown).

Administration of *E. coli* endotoxin increased the activity of iNOS (by  $491\pm6\%$ , n=5, P<0.001) and decreased indomethacin-induced lesion-formation (by  $68\pm7\%$ , n=5, P<0.01) in the stomach of ovary-intact female rats. Data are shown in Fig. 3. We found that cNOS activity did not change throughout this experimental series (data are not shown).

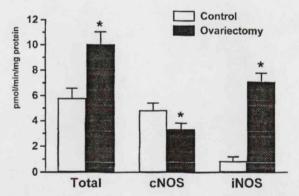


Fig. 1. Total, constitutive and inducible nitric oxide synthase (cNOS and iNOS, respectivelly) activity (assessed by the citrulline assay, and expressed as pmol/min/mg protein) in the stomach of control and ovariectomized female rats. Data are shown as the mean $\pm$ S.E.M., where (n) is at least 10 measurements in a group, and where statistical significance is given as \*P<0.05 between ovariectomized and control groups.

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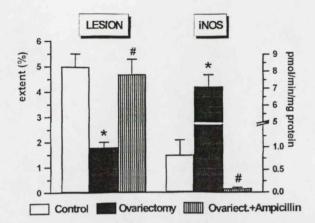
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Fig. 2. Indomethacin (50 mg/kg, s.c., 4 h)-induced lesion formation (expressed as the per cent of the total gastric mucosal surface), and inducible nitric oxide synthase (iNOS) activity (assessed by the citrulline assay, and expressed as pmol/min/mg protein) in the stomach of control (ovary-intact), ovariectomized and ampicillin-treated (800 mg kg<sup>-1</sup>, p.o., 3 days) ovariectomized female rats. Data are shown as the mean  $\pm$  S.E.M., where (n) is at least five rats in a group, and where statistical significance is given as \*P<0.001 between ovariectomized and control groups;  ${}^{\mu}P$ <0.05 between ampicillin-treated ovariectomized and ovariectomized groups.

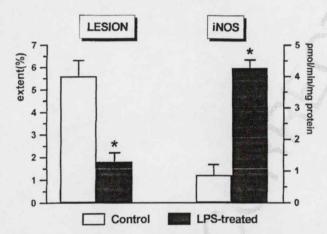


Fig. 3. Indomethacin (50 mg/kg, s.c., 4 h)-induced lesion formation (expressed as the per cent of the total gastric mucosal surface), and inducible nitric oxide synthase (iNOS) activity (assessed by the citrulline assay, and expressed as pmol/min/mg protein) in the stomach of control (ovary-intact), and exogenous bacterial endotoxin (*E. coli* lipopolysaccharide (LPS) 0111:B4, 3 mg/kg, i.v., 5 h)-treated, ovary-intact female rats. Data are shown as the mean  $\pm$  S.E.M., where (n) is at least five rats in a group, and where statistical significance is given as \*P < 0.001 between LPS-treated and control groups.

#### 4. Discussion

Our present results are in agreement with earlier observations, where total NO synthase activity has increased in the stomach following ovariectomy [12]. In these oestrogen-deficient rats, the elevation of iNOS activity was shown to be responsible for the significant

increase of total NO synthase activity, since, in the gastric tissue, we found a decreased eNOS activity which corresponds with our recent findings in the heart and aorta [11]. Thus, oestrogen-deficiency down-regulates eNOS in the stomach, too, similarly that found by others in the vascular tissue [16,20].

The decrease of physiological cNOS activity is known to initiate an increase in neutrofil infiltration to the vascular endothelium, platelet aggregation, an impaired vasodilatation and an elevation of vascular permeability, processes which attenuate blood supply towards the gastric tissue [9,21]. Moreover, a reduction in cNOS activity decreases mucus level in the stomach [12,14]. 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, which may trigger iNOS activation. Indeed, administration of the wide spectrum antibiotic ampicillin blocked the expression of iNOS, even under the control iNOS activity.

A controlled elevation in gastric NO level, originated from an increased endogenous production or exogenous administration, defends the stomach against injury [8,12,15,18,19,23]. In our present study, we showed that administration of exogenous bacterial endotoxin protected the gastric mucosa against indomethacin-induced ulceration and increased iNOS activity in ovary-intact rats, which confirms earlier observations, where different ulcer models were used [19,23]. Moreover, other investigators demonstrated that neutrophil depletion caused an enhanced generation of endogenous NO, which improved the defensive mechanisms of the stomach [18]. Finally, NO administered exogenously by using NO donors, significantly ameliorated gastric mucosal damage provoked by various ulcerogenic agents [8,13]. The earlier-described findings give further support for the protective role of an increased NO synthase enzyme activity by the expression of iNOS, and, in addition, suggests that the endotoxin component of endogenous gram-negative bacteria is responsible for iNOS activation resulting gastric mucosal protection.

In summary, we suppose that gastric protection against ulcerogenic stimuli following ovariectomy is mediated by an increased total NO synthase enzyme activity, although the reduction of eNOS activity has been shown in the stomach. This accords to the cardio-vascular experience that in the oestrogen-deficient state the down-regulation of eNOS develops [11,20]. However, in the case of the aorta and the heart, iNOS activity did not change following ovariectomy [11,20], while in the stomach we demonstrated an enhanced iNOS activity. We suppose that in the oestrogen-deficient state, endogenous bacteria trigger the activation of iNOS, which leads to increased NO production in the stomach, a process improving gastric mucosal defence.

#### Acknowledgements

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Szepes Z., Kiss J., Lamarque D., Moran A.P., Nemcsik J., <u>Morschl É.,</u> László F., Whittle B.J.R.

Attenuation of Helicobacter pylori endotoxin provoked rat intestinal inflammation by selective inhibition of the inducible nitric oxide synthase.

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# Attenuation of *Helicohacter pylori* endotoxin-provoked rat intestinal inflammation by selective inhibition of the inducible nitric oxide synthase

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#### Abstract

We studied the actions of purified *Helicobacter pylori* endotoxin (3 mg kg<sup>-1</sup>, i.v.) on rat intestinal vascular permeability (assessed by the radiolabelled human serum albumin leakage technique) and on nitric oxide synthase induction (assessed by the circulline assay) 4 h later. We found increased albumin leakage and expression of the inducible nitric oxide synthase in jepinom and colon, effects reversed by a selective inducible nitric oxide synthase inhibitor N-(8-(aminomethyl)benzyl)-acetamidine (1400W; 0.2-1 mg kg<sup>-1</sup>, s.c., concurrently with endotoxin). Thus, *H. pylori* endotoxin seems to be capable of provoking an inflammatory response in the rat intestinal tissue. Transmucosal invasion or/and surface translocation of *H. pylori* or its breakdown products might possibly attenuate jejunal and colonic mucosal barrier function, a process mediated by the expression of the inducible intric oxide synthase. © 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Helicobacter pylori endotoxin; Intestinal inflammation; Inducible nitric oxide synthase

#### 1. Introduction

Melicobacter pylori is an acid-resistant bacterium, which can grow only at low pH, predominantly in the antral part of the stomach. All over the world, the incidence of H. pylori infection is high, e.g. it approaches 50-80% in European adults. The pathological involvement of H. pylori is known in gastroduodenal inflammation and duodenal ulceration, and it might participate in the generation of gastric malignancies too [6]. Although the clinical impact of a better understanding of how H. pylori affects gastrointestinal mucosa is high, only a few investigations had been carried out by using its purified endotoxin for exploring its local or/and systemic effect. In chronic inflammatory diseases of the gut (e.g. in inflammatory bowel diseases), the overproduction of nitric oxide (NO) by the expres-

In the present study, we investigated the relation between the expression of iNOS and intestinal inflammation following systemic administration of a purified *H. pylori* endotoxin [5].

#### 2. Materials and methods

#### 2.1. Treatments

We used male Wistar rats (230-250 g). They were fasted overnight, but received water ad libitum. Under transient ether anaesthesia, purified *II. pylori* endotoxin (from Professor Moran's laboratory, Ireland; 3 mg kg<sup>-1</sup>) was administered intravenously. In a separate experiment, we administered a bisisothiourea derivative selective iNOS inhibitor (from Wellcome Research Laboratories, N-(8-(aminomethyl)-benzyl)acetamidine

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sion of the inducible NO synthase enzyme (iNOS) is cytotoxic, and has a pathological impact [7]. It increases vascular permeability, adhesion of neutrophils to the vascular endothelium and leads to vasocongestion [4].

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[1400W], 0.2-1 mg kg<sup>-1</sup>, s.c.; [2]) concurrently with endotoxin. For the measurement of vascular permeability or iNOS enzyme activity, segments of the jejunum and colon were removed from standard sites 4 h following treatments.

#### 2.2. Intestinal nitric oxide synthase enzyme activity

In this experiment, we used untreated (control) and II. pylori endotoxin (3 mg kg<sup>-1</sup>, i.v.)-treated groups alone or with 1400W (1 mg kg<sup>-1</sup>, s.c., concurrently with endotoxin) administration. In jejunal and colonic tissues, we measured the calcium-dependent constitutive NO synthase (cNOS) and the calcium-independent iNOS activity by the citrulline assay as described previously [1,2].

#### 2.3. Intestinal vascular permeability

In separate groups of rats, as a measure of inflammation, we examined jejunal and colonic vascular permeability by using the radiolabelled human serum albumin leakage technique as has been established in earlier studies [1]. We used *H. pylori* endotoxin (3 mg kg<sup>-1</sup>, i.v.)-treated groups alone or with 1400W (0.2–1 mg kg<sup>-1</sup>, s.c., concurrently with endotoxin) administration.

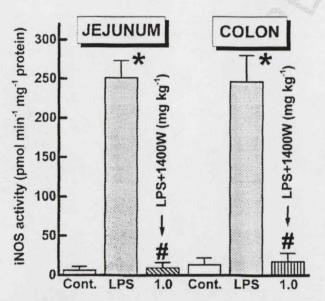


Fig. 1. Expression of iNOS (described in pmol min<sup>-1</sup> mg<sup>-1</sup> protein units) in the rat jejunal and colonic tissue 4 h after the administration of purified *Helicobacter pylori* endotoxin (LPS; 3 mg kg<sup>-1</sup>, i.v.), and its reversal by the selective iNOS inhibitor 1400W (1 mg kg<sup>-1</sup>, s.c., concurrently with LPS). Data are expressed as mean $\pm$ S.E.M., where n=4 rats in a group. \*P<0.05 means significant increase in iNOS activity compared with the untreated control (Cont.) group; #P<0.05 means significant decrease in iNOS activity compared to the LPS alone group.

#### 2.4. Chemicals

We obtained [1251]human serum albumin from IZINTA (Budapest, Hungary) and L-[U-14C]arginine monohydrochloride from Amersham International (UK). All other compounds were from Sigma.

#### 2.5. Statistics

Throughout our studies, the results were expressed as mean  $\pm$  S.E.M. of (n) rats in a group. Data were analysed with the Tukey-Kramer multiple comparisons test, where P < 0.05 was taken as significant.

#### 3. Results

#### 3.1. Expression of intestinal inducible nitric oxide synthase

Administration of *H. pylori* endotoxin led to the expression of iNOS in the jejunum and colon (n-4, P<0.001) 4 h later, an effect which was abolished by the administration of 1400W iNOS (p-4, P<0.001) in both intestinal tissues (Fig. 1). Basal cNOS activities remained unchanged throughout the experiments (n-4, data are not shown).

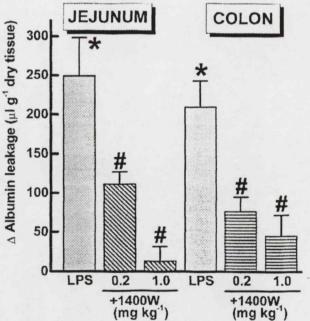


Fig. 2. Provocation of jejunal and colonic albumin leakage (expressed as  $\mu$ l albumin  $g^{-1}$  dry tissue) by the administration of a purified *Helicobacter pylori* endotoxin (LPS; 3 mg kg<sup>-1</sup>, i.v.) 4 h later, and its dose-dependent attenuation by the concurrent administration of a selective iNOS inhibitor (1400W, 0.2–1 mg kg<sup>-1</sup>, s.c.). Data are expressed as mean  $\pm$  S.E.M., where n = 4-6 rats in a group. \*P < 0.05 means significant increase in albumin leakage; "P < 0.05 means significant decrease in albumin leakage compared to the LPS alone group.

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#### 3.2. Intestinal vascular permeability

Administration of *H. pylori* endotoxin (3 mg kg<sup>-1</sup>, i.v.) alone provoked significant small and large intestinal albumin leakage in both tissues 4 h later (Fig. 2). Concurrent administration of the selective inhibitor of iNOS, 1400W with *H. pylori* endotoxin dose-dependently attenuated jejunal and colonic albumin leakage after 4 h (83 $\pm$ 5% and 94 $\pm$ 3% maximal protection, respectively; n=4-6, P<0.01) as demonstrated in Fig. 2.

#### 4. Discussion

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Our present results confirm previous observations, when a II. pylori extract was administered intravenously, and the expression of iNOS in the duodenal part of the small intestine and in its epithelial cells had been found [3]. The expression of iNOS correlated with epithelial cell death, and could be reversed by the adminisof the non-selective iNOS inhibitor, dexamethasone, and by the treatment with NG-nitro-Larginine methyl ester, a non-specific NO synthase blocker at the time of the expression of iNOS [3]. Thus, II. pylori extract could damage the intestinal mucosa by its direct toxic effect on epithelial cells, which is mediated by iNOS. The vascular injury correlating with iNOS expression, and their reversal by the selective iNOS inhibitor, 1400W following the challenge with H. pylori purified endotoxin in our study suggests that, besides the damage of epithelial cells, microcirculatory dysfunction can also participate in H. pylori-related inflammation. Morever, the induction of NO synthase and microvascular leakage in the jejunum and colon might focus the impact of H. pylori endotoxin-provoked mucosal injury in other parts of the gastrointestinal tract.

In conclusion, H. pylori endotoxin seems to be capable of provoking a inflammatory response in the rat intestinal tissue. Transmucosal invasion or/and surface

translocation of *H. pylori* or its breakdown products might attenuate the nucesal barrier function of the jejunum and colon. This process is mediated by iNOS. Thus, the clinical implication of selective inhibitors of iNOS might have potential therapeutic benefit in the prevention and treatment of *H. pylori*-associated gastrointestinal nucesal inflammation.

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### Whittle B.J.R., Morschl É., Pozsár J., Moran A.P., László F.

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**PERGAMON** 

Physiology Paris

Helicobacter pylori lipopolysaccharide provokes iNOS-mediated acute systemic microvascular inflammatory responses in rat cardiac, hepatic, renal and pulmonary tissues

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#### Abstract

We have examined the effects of intravenous administration of a purified lipopolysaccharide (LPS) from Helicobacter pylori (3 mg kg<sup>-1</sup>, i.v.) on rat vascular permeability, assessed by the radiolabelled human serum albumin leakage technique in the heart, kidney, liver and lung 4 h after challenge. An increased vascular permeability in cardiac, renal, hepatic and pulmonary tissues after challenge was determined. The albumin leakage observed in all these organs could be prevented by the selective inducible nitric oxide synthase inhibitor, N-(8-(aminomethyl)benzyl)-acetamidine (1400W; 0.2-1 mg kg<sup>-1</sup>, s.c.) administration concurrently with LPS. Thus, H. pylori LPS can provoke a microvascular inflammatory response in the rat cardiac, renal, hepatic and pulmonary tissues, actions mediated through the activation of the inducible nitric oxide synhase isoenzyme. © 2001 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Helicobacter pylori endotoxin; Heart; Lung; Kidney; Liver; Inflammation; Inducible nitric oxide synthase

#### 1. Introduction

Following the identification of the *Helicobacter pylori* organism [7], an acid-resistant bacterium growing predominantly in the antrum of the human stomach, it has became clear that it plays a role in the development of chronic atrophic gastritis, gastric malignancies, especially in mucosa-associated lymphatic tissue (MALT) lymphomas, and gastroduodenal ulceration [1,10]. In addition to its gastroduodenal pathogenic role, experimental and clinical data suggest that *H. pylori* infection may play a role in numerous diseases of organs in addition to the gut [6,9,11], although no clear-cut correlation between *H. pylori* infection and these disease states have been obtained.

The purification of lipopolysaccharide (LPS) from H. pylori has allowed a better insight for exploring the potential pathologic processes related to the bacterium

and its products [8]. The excess generation of nitric oxide (NO) by the activation of the inducible NO synthase enzyme (iNOS) is triggered by bacterial endotoxins. This overproduction of NO has cytotoxic potential and provokes microvascular inflammatory reactions. Recent studies have shown that the LPS from H. pylorican provoke cytotoxicity in rat duodenal epithelial cells, an effect involving the expression of iNOS and superoxide production, implicating a role of peroxynitrite [4]. Therefore, in our present study, we have investigated whether systemic administration of a purified H. pylori\_LPS leads to microvascular dysfunction in various extraintestinal organs of the rat and the actions of selective inhibitors of iNOS.

#### 2. Materials and methods

#### 2.1. Treatments

Male Wistar rats (230-250 g) were fasted overnight, but received water ad libitum. Under transient ether

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anaesthesia, purified LPS from *H. pylori* (3 mg kg<sup>-1</sup>) was administered intravenously. In a separate experiment, we administered a bisisothiourea derivative selective iNOS inhibitor (GlaxoWellcome, N-(8-(aminomethyl)benzyl)acetamidine [1400W], 0.2-1 mg kg<sup>-1</sup>, s.c.; [2]) concurrently with the LPS. For the measurement of vascular permeability, segments of the left ventricle of the heart, pulmonary parenchyma, liver and kidney were removed from standardised sites, 4 h following treatments.

Vascular permeability in the heart, kidney, liver and lung was determined by using the radiolabelled human serum albumin leakage technique, established in earlier studies [5].

#### 2.2. Chemicals

[125I]human serum albumin was from IZINTA (Budapest, Hungary) and L-[U-14C]arginine monohydrochloride was from Amersham International (UK). All other compounds were from Sigma Chemical Company.

#### 2.3. Statistics

The results were expressed as mean  $\pm$  S.E.M. of (n) rats in a group. Data were analysed with the Tukey-Kramer multiple comparisons test, where P < 0.05 was taken as significant.

#### 3. Results

3.1. Cardiac, renal, hepatic and pulmonary vascular permeability, and their attenuation by the selective inhibition of the inducible nitric oxide synthase

Administration of *H. pylori* LPS (3 mg kg<sup>-1</sup>, i.v.) alone provoked significant cardiac, renal, hepatic and pulmonary albumin leakage, determined 4 h later (Fig. 1).

Concurrent administration of the selective inhibitor of iNOS, 1400W with H. pylori LPS, dose-dependently attenuated albumin leakage after 4 h in the rat heart, kidney, liver and lung, with maximal inhibition of  $91\pm2\%$ ,  $73\pm7\%$ ,  $93\pm6\%$  and  $98\pm\%$  maximal protection, respectively  $(n=4-6,\ P<0.01)$ . The results are demonstrated in Fig. 1.

#### 4. Discussion

In our present study, we have found increased microvascular permeability to albumin in the rat heart, liver, kidney and lung, 4 h after the administration of purified LPS from *H. pylori*. Such findings agree with earlier studies, where a purified *Escherichia coli* LPS lead to

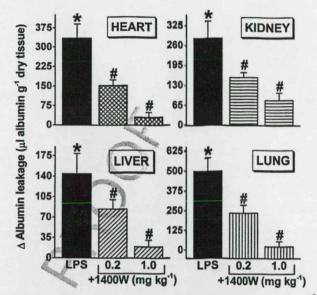


Fig. 1. Albumin leakage (expressed as ml albimn  $g^{-1}$  dry tissue) in the rat heart, kidney, liver and lung 4 h following the administration of purified *Helicobacter pylori* endotoxin (LPS; 3 mg kg<sup>-1</sup>, i.v.), and its reversal by the selective iNOS inhibitor 1400W (0.2–1 mg kg<sup>-1</sup>, s.c., concurrently with LPS). Data are expressed as mean  $\pm$  S.E.M., where n=4-6 rats in a group. \*P<0.05 means a significant increase in albumin leakage; \*P<0.05 means a significant decrease in albumin lekage compared to the LPS group.

similar systemic vascular inflammatory actions under comparable experimental conditions [2,5]. In the present study, we have found that the selective inhibitor of iNOS, 1400W attenuated the microvascular responses in these tissues induced by *H. pylori* LPS, which supports the pathogenic involvement of iNOS. Previous studies have shown that the cytotoxic effects on rat duodenal epithelial cells following challenge with this *H. pylori* LPS could be abolished by 1400W [4]. Likewise, the actions of *E. coli* LPS on vascular permeability could also be reversed by 1400W [2] or by non-selective NO synthase inhibitors when administered at the time of expression of iNOS [5].

In recent years, the etiological role of infections, including H. pylori, has been discussed in the pathogenesis of diseases such as atherosclerosis and coronary heart disease [6]. Furthermore, the association of H. pylori infections and chronic liver diseases, including cirrhosis and chronic cholangitis, has been reported, but no causal relationship has been shown [11]. In addition, H. pylori infection may be involved in the etiology of pulmonary and renal MALT lymphoma [3,12], bronchiectasia [13] and membranous nephropathy [9]. However, more data are needed to establish correlation between the bacterium and the disease. Our present results suggest that the purified LPS from H. pylori is capable of provoking an iNOS-mediated systemic microvascular inflammatory response. Thus, transmigration of the bacteria into gastro-duodenal tissue and

liberation of its LPS into the systemic circulation may provide one pathogenic process in these diseases, through expression of iNOS.

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