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L-GLUTAMINE INHIBITS THE RELEASE OF ENDOTHELIUM-DERIVED RELAXING FACTOR FROM THE RABBIT AORTA

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Summary. The present study investigates the effect of the amino acid L-glutamine (L-Gln) on the release of endothelium-derived relaxing factor (EDRF) from the luminally perfused rabbit aorta and on endothelium-dependent relaxations of rabbit aortic strips. L-Gln (200 μM) had no effect on the acetylcholine (Ach)-induced release of EDRF from freshly prepared aortic tissues. The concentration of L-arginine (L-Arg) in endothelial cells isolated from these aortae was approximately 4 mM, as determined by HPLC analysis. After an initial equilibration period of 2 h and two consecutive infusions of Ach (55 μM for 15 min) at 2 and 3 h, L-Arg levels fell by 62 $\pm$ 14% (n=4). Under these conditions, L-Gln (200 μM) but not D-Gln (200 μM) inhibited the release of EDRF by 50 $\pm$ 4 % (n=7). This effect of L-Gln was partially reversed by infusions of L-Arg (500 μM) but not D-Arg (500 μM). L-Gln (200 μM) but not D-Gln (200 μM) potentiated the inhibitory effect of N^{ω}-nitro-L-arginine (30 μM), an inhibitor of EDRF biosynthesis, on Ach-induced relaxations of rabbit aortic strips, whereas L-Gln alone had no effect. Thus, L-Gln inhibits the release of EDRF from intact blood vessels presumably by interfering with the generation of L-Arg by the endothelium.

Endothelium-derived relaxing factor (EDRF) has been identified as nitric oxide or a closely related molecule derived from the guanidino group of L-arginine (L-Arg) (1-3). Cultured bovine aortic endothelial cells can generate L-Arg from an intracellular source (4) and maintain their intracellular L-Arg levels during periods of prolonged EDRF release (5). Both the generation of L-Arg and the release of EDRF are inhibited by L-glutamine (L-Gln) (6), supporting the hypothesis that the generation of L-Arg in endothelial cells is closely linked to the release of EDRF (7). As these studies were performed in cultured endothelial cells, we wanted to investigate whether the endothelium of intact blood vessels has a similar L-Arg dependent and L-Gln sensitive pathway of EDRF biosynthesis.

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MATERIALS AND METHODS

Materials. L-Arg and D-Arg, L-Gln and D-Gln, N^{ω}-nitro-L-arginine methyl ester (NO_2Arg), indomethacin, L-phenylephrine, superoxide dismutase (SOD), atropine hydrochloride and acetylcholine hydrochloride (Ach) were obtained from Sigma Chemical Co. (Poole, UK). 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) was a generous gift of Dr. J. Pike, The Upjohn Company (Kalamazoo, MI, USA). Glyceryl trinitrate (GTN, Nitronal^R) was supplied by Lipha Pharmaceuticals Ltd (West Drayton, UK), collagenase type I by Worthington Biochemical Corporation (Freehold, NJ, USA), and Hanks' Balanced Salt Solution (HBSS) by Flow Laboratories (Irvine, UK). Sodium pentobarbitone (Sagatal^R) was obtained from May & Baker Ltd. (Dagenham, UK) and lignocaine hydrochloride from Antigen Ltd. (Roscrea, Ireland). All other reagents were of the highest quality commercially available.

Luminally perfused rabbit aorta (RbA). Male New Zealand White Rabbits (2.0-2.5 kg) were anaesthetized with sodium pentobarbitone (Sagatal^R; 60 mg/kg i.v.) and exsanguinated via the carotid artery. The aorta was prepared for the perfusion as described previously (8) and perfused at 5 ml/min with warmed (37^{O}C), oxygenated (95%O_2/5%CO_2) Krebs' solution (pH 7.4) containing indomethacin (5.6 μM) and SOD (10 u/ml). The composition of the Krebs' solution was as follows (in mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.17; $CaCl_2 \cdot 6H_2O$, 2.5; $NaHCO_3$, 25 and glucose, 5.6. The effluent from the aorta superfused a cascade (9) of four spirally cut rabbit aortic strips which were mechanically denuded of endothelium and equilibrated with a preload of 2 g for 1-2 h. They were preconstricted by infusions of U46619 (30-60 nM) or L-phenylephrine (100 nM). The effluent from the aorta reached the consecutive rabbit aortic strips after 1, 4, 7 and 10 s, respectively. Drugs were either injected directly over the detector tissues (OT) or through the aorta (TA). GTN (22-132 pmoles, OT) was used to calibrate the responses of the detector tissues which were electronically adjusted to give similar relaxations to a standard dose. Relaxations were recorded by using auxotonic levers attached to Harvard heart/smooth muscle transducers and displayed on a Watanabe WR 3310 multichannel pen recorder. The aortae were perfused for a total of 4-6 hours. After an initial equilibration period of 2 h, 3-5 infusions of Ach (55 μM TA for 15 min) were made at hourly intervals during the course of the experiment. The direct effect of Ach on the detector tissues was antagonized by atropine (3.5 μM) infused OT throughout the experiment. Initially, 2 control infusions of Ach (55 μM) were made TA followed by L-Gln or D-Gln (200 μM, TA) infusions given 20 min before the third Ach infusion and maintained throughout the experiment. L-Arg or D-Arg (500 μM, TA) infusions were started 20 min before the fourth Ach infusion and maintained throughout the experiment. All drugs were infused OT before being infused TA. Ach-induced relaxations were expressed as a ratio of the area under the curve representing Ach-induced EDRF release in comparison to the mean of two areas under the curves representing the response to GTN (OT) before and after each ACh infusion.

Organ bath experiments. Rabbits were anaesthetized and exsanguinated as described above. The thoracic aorta was carefully removed, cleaned of fat and connective tissue and cut into rings of 5 mm width. The rings were cut open and aortic strips were mounted in 20 ml organ bath filled with warmed (37 ^{O}C), oxygenated (95% O_2 / 5% CO_2) Krebs' solution containing indomethacin (5.6 μM) and EDTA (50 μM). The strips were equilibrated under a resting tension of 2 g for 90 minutes and the Krebs' solution was changed every 15 minutes. The tissues were preconstricted with noradrenaline (NA; 6.3 - 35.2 nM) to 1 g of tension. Changes in isometric tension were measured with Biegestab K30 type 351 transducers (Hugo Sachs Elektronik) attached to MK II transducer coupler (Z.T.S., London, UK) and recorded with a Watanabe WR 3310 recorder. Relaxations of more than 80% to Ach (1 μM) confirmed the presence of intact endothelium. Tissues were then washed, preconstricted with NA and a cumulative dose-response curve to Ach (5.5 nM-5.5 μM) was established. Drugs were added to the organ bath at a standard volume of 100 μl and incubated for 45 min (fresh Krebs' solution containing these compounds was added every 15 min). A second dose-response curve to Ach was then established and results expressed as % relaxation of induced tone.

HPLC analysis of L-Arg and L-citrulline (L-Cit) concentrations in endothelial cells isolated from the luminally perfused RbA. Amino acid concentrations were measured in different stages of perfusion: i) after 5 min (necessary to wash out blood), ii) after two infusions of Ach TA (*ie* prior to the L-Gln infusion) and iii) after the third infusion of Ach TA, in the absence ("time control" experiments) or presence of L-Gln. The aorta was removed from the cascade, cannulated from both sides, filled with collagenase (1 mg/ml in HBSS), placed into a Petri dish and incubated at 37 ^{o}C. After 20 min, the detached endothelial cells were rinsed with HBSS into a small tube and centrifuged at 1,000 rpm for 5 min. The supernatant was aspirated, the cells washed with HBSS and centrifuged again at 1,000 rpm for 5 minutes. They were then resuspended in HBSS (200 μl), an aliquot (50 μl) was taken for cell counting and assessment of viability (Trypan blue exclusion), and the remaining cells (150 μl) were extracted with methanol (350 μl). After centrifugation for 15 min at 10,000 rpm (to facilitate deproteinization of the sample), the supernatant was stored at -20 ^{o}C before HPLC analysis. Reversed phase HPLC/fluorescence detection analysis for quantification of the endogenous amino acids was performed as described previously (4). The freshly harvested cells were morphologically identified as endothelial cells based on their cobblestone-like appearance after culturing in Petri dishes.

Statistics. All values represent mean $\pm$ s.e.m. from n individual experiments. Statistical differences between groups were assessed by Student's *t*-test for unpaired data and the dose-response curves to Ach were analysed by two way analysis of variance (ANOVA). A *p*-value of less than 0.05 was taken as significant.

RESULTS

The effect of L-Gln on EDRF release from the luminally perfused RbA. In freshly-prepared aortae, L-Gln infusions (200 μM) did not significantly inhibit Ach-induced EDRF release (n=3), (data not shown). Therefore, we infused Ach twice (TA) for 15 minutes prior to the L-Gln infusion (TA) to deplete endothelial L-Arg levels. In time control studies, relaxations elicited by the third infusion of Ach were not different from relaxations elicited by the second Ach infusion. However, when the third infusion of Ach was administered concomitantly with L-Gln,EDRF release was inhibited by 50 $\pm$ 4% (n=7; Fig. 1). D-Gln (200 μM) did not inhibit Ach-induced EDRF release (n=3)

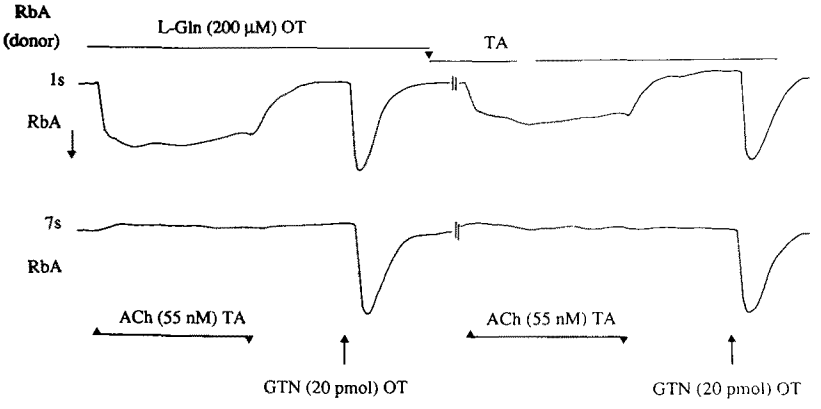

Fig. 1. L-Gln inhibits the ACh-induced release of EDRF from the luminally perfused RbA. The figure represents a typical trace from n=7 experiments. As described in the methods section, Ach was infused twice prior to the infusion in the presence of L-Gln or D-Gln. The arrows indicate bolus injections of GTN (44 pmoles) used to calibrate the responses of the detector tissues and to normalize Ach-induced relaxations.

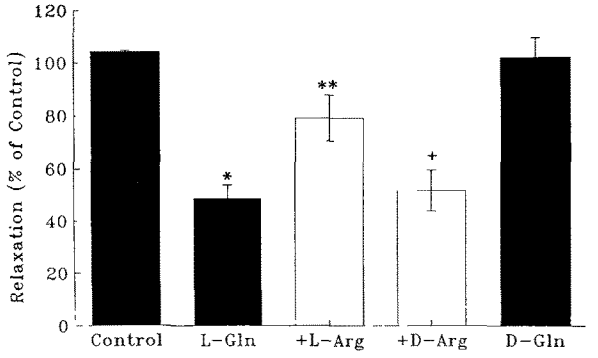

Fig. 2. L-Gln inhibits the ACh-induced release of EDRF from the luminally perfused RbA. The release of EDRF induced by the second infusion of Ach was taken as 100 %. L-Gln (n=7) or D-Gln (n=3) were administered during the third and L-Arg (n=4) or D-Arg (n=3; illustrated as +L-Arg and +D-Arg) during the fourth challenge with Ach. Control (n=3) represents EDRF release evoked by the third infusion of Ach performed in the absence of L-Gln. Values represent means ± s.e.m. from the number of experiments indicated. *,** and + denote significant differences ($p<0.05$) from control (*), from L-Gln alone (**) and from L-Gln plus L-Arg (+).

(Fig. 2). When infused OT, L-Gln or D-Gln (200 μM) did not interfere with the detection of EDRF and did not release EDRF when infused TA alone. Infusions of L-Arg (500 μM, TA) partially reversed the inhibitory effect of L-Gln (21 ± 2% inhibition; n=4), whereas D-Arg (500 μM, TA) had no effect (n=3; Fig. 2). When infused OT, L-Arg or D-Arg (500 μM) did not influence the tone of the detector tissues and did not release EDRF when infused TA alone.

The effect of perfusion and infusions of Ach and L-Gln on L-Arg and L-Cit levels in aortic endothelial cells. The initial concentration of L-Arg and L-Cit in endothelial cells isolated from rabbit aorta were 4.1 ± 1.6 and 3.7 ± 1.6 mM (n=4; Table 1). After two infusions of Ach, L-Arg and L-Cit levels fell by 62 ± 14% and 52 ± 17% (n=4) and by 76 ± 13% and 88 ± 4% after three infusions (n=3). When L-Gln was present during

Table 1. Comparison of L-Arg and L-Cit concentrations in freshly isolated endothelial cells during different stages of perfusion

Time	L-Arg (mM)	L-Cit
initial (4)	4.06 ± 1.63	3.71 ± 1.58
2 Ach infusions (4)	1.54 ± 0.55	1.78 ± 0.63
3 Ach infusions (3)	0.98 ± 0.51	0.44 ± 0.15
3 Ach infusions plus L-Gln (6)	0.65 ± 0.23	0.74 ± 0.38

For experimental details refer to *Materials and Methods*.
Values represent mean ± s.e.m. with the number of aortae in parenthesis.

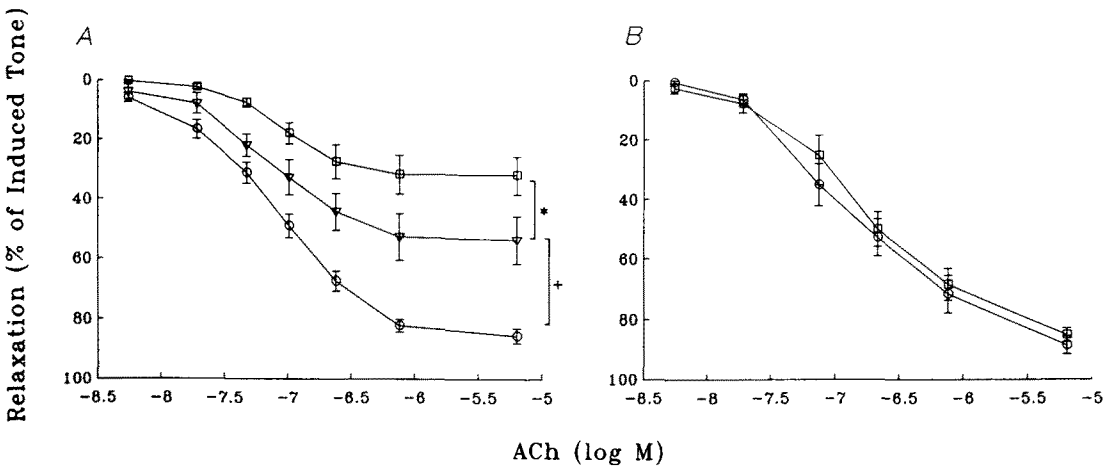

Fig. 3. (A) Comparison of the inhibitory effect of NO_2Arg (30 μM), in the presence (open squares) or absence (open triangles) of L-Gln (200 μM), on Ach-induced, endothelium-dependent relaxations of rabbit aortic strips (n=6-8 from 5 rabbits). Control responses are represented by open circles. (B) L-Gln (200 μM) alone did not influence relaxations evoked by Ach (n=5 from 3 rabbits). D-Gln (200 μM) when given together with NO_2Arg (30 μM) did not affect Ach-induced relaxations (n=4 from 3 rabbits; not shown). * and + denote significant differences ($p<0.05$ by ANOVA) between control and NO_2Arg in the absence (+) or presence of L-Gln (*).

the third infusion of Ach, the intracellular levels of L-Arg and L-Cit were reduced by 84 $\pm$ 6% and 80 $\pm$ 10% (n=6).

The effect of L-Gln on Ach-induced, endothelium-dependent relaxations of rabbit aortic strips. Ach induced dose-dependent relaxations of preconstricted rabbit aortic strips which were dependent on the presence of an intact endothelium. Incubations of the tissues with NO_2Arg (30 μM), an inhibitor of EDRF biosynthesis (10), resulted in a moderate inhibition of the response to Ach. This effect of NO_2Arg was significantly potentiated in the presence of L-Gln (200 μM; Fig. 3) but not D-Gln (200 μM; data not shown). L-Gln alone had no effect on endothelium-dependent relaxation (Fig. 3).

DISCUSSION

The present study demonstrates that L-Gln stereospecifically inhibits the Ach-induced release of EDRF from the luminally perfused aorta of the rabbit. The inhibitory effect of L-Gln was partially reversed by co-infusions of L-Arg but not D-Arg, suggesting that L-Gln interferes with EDRF biosynthesis. Moreover, L-Gln but not D-Gln potentiated the inhibitory effect of NO_2Arg on endothelium-dependent relaxations of the rabbit aorta. This indicates that L-Gln may either affect the generation of L-Arg necessary for EDRF biosynthesis and/or exert a regulatory effect on the binding of NO_2Arg to the EDRF-forming enzyme(s). These findings are an extension of our previous work showing that L-Gln inhibits the generation of L-Arg and the release of EDRF from bovine aortic endothelial cells cultured in the presence or absence of L-Arg (6).

It is important to note that L-Gln inhibited the release of EDRF only from perfused aortae which were repeatedly challenged with Ach but not from freshly prepared tissues, probably due to their high endothelial L-Arg concentration (4,11). Thus, it is conceivable that the inhibitory effect of L-Gln may only be visible in circumstances where the endothelium synthesizes L-Arg *de novo*. In cultured endothelial cells, the inhibition of EDRF release by L-Gln is associated with a blockade of the generation of L-Arg (6) which may be derived from L-Cit via an Arg-Cit cycle (4). However, the changes in intracellular L-Arg or L-Cit in freshly isolated endothelial cells were not significantly different in the absence or presence of L-Gln, but there was a trend towards a reduction in L-Arg and an accumulation of L-Cit. Possible reasons for this discrepancy may be related to the technical difficulties, *eg* the isolation procedure, or to the inability to discern different intracellular amino acid pools with the method employed. Alternatively, the mechanism by which L-Gln inhibits the release EDRF release from non-cultured endothelial cells, *ie* the endothelium is in close contact with the underlying smooth muscle, may be more complex than in cultured endothelial cells.

We conclude that the biosynthesis of EDRF by the intact vessel wall is inhibited by L-Gln when the endothelium is deficient in L-Arg and forced to generate L-Arg from an intracellular source. Thus, L-Gln may be considered as an endogenous inhibitor of the release of EDRF *in vivo* under conditions where the availability of L-Arg is rate-limiting for EDRF biosynthesis.

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