Inhibition by L-glutamine of the release of endothelium-derived relaxing factor from cultured endothelial cells

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L-Glutamine (0.02–2 mM) but not D-glutamine (0.2 mM and 2 mM) inhibited the release of endothelium-derived relaxing factor (EDRF) from bovine aortic endothelial cells cultured in the presence or absence of L-arginine. Inhibition was maximal at a concentration of 200 μ M, and was reversed by L-arginine (50 μ M) but not D-arginine (100 μ M). L-Glutamic acid (2 mM) or ammonium chloride (1 mM), putative products of the metabolism of L-glutamine in endothelial cells, had no effect on EDRF release. L-Glutamine (0.2 mM and 2 mM) but not D-glutamine (2 mM), L-glutamic acid (2 mM) or ammonium chloride (1 mM) also inhibited the generation of L-arginine in endothelial cells. Thus, L-glutamine inhibits EDRF release by preventing the generation of L-arginine.

Introduction 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) (Palmer *et al.*, 1988a). The release of EDRF from cultured endothelial cells is inhibited by L-Arg analogues, such as N^G-monomethyl-L-arginine, an effect that is reversed by L-Arg but not D-Arg (Palmer *et al.*, 1988b). However, L-Arg elicits little or no relaxation of freshly isolated vascular preparations (Thomas *et al.*, 1989), probably due to their high L-Arg content (Hecker *et al.*, 1990). These findings indicate that L-Arg is the endogenous substrate of EDRF biosynthesis and that its availability is rate-limiting for EDRF release.

EDRF release from cultured endothelial cells appears to depend on the generation of L-Arg (Mitchell *et al.*, 1990a). L-Arg is generated when the cells are transferred from culture medium to Krebs solution, suggesting an inhibitory factor to be present in the medium. Interestingly, this transfer coincided with a rapid fall of L-glutamine (L-Gln) (Mitchell *et al.*, 1990b). As L-Gln was also a major constituent of the culture medium, we investigated whether L-Gln inhibits the release of EDRF from cultured endothelial cells.

Methods Endothelial cells cultured from bovine aorta were grown on Cytodex-3 microcarrier beads as described (de Nucci *et al.*, 1988). After reaching confluence, they were cultured for another 24 h in either Dulbecco's modified Eagle's medium (DMEM) containing 0.6 mM L-Arg and 4 mM L-Gln or Eagle's minimum essential medium (MEM) containing 2 mM L-Gln but not L-Arg. The cells were packed into a jacketed chromatography column (approx. 6×10^7 cells/column) and perfused at 5 ml min^{-1} with warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs solutions containing indomethacin (5.6 μ M) and superoxide dismutase (10 U ml⁻¹). The effluent from the column superfused a cascade (Vane, 1964) of 4 spirally cut rabbit aortic strips (RbA) denuded of endothelium and precontracted with 30 nM 9,11-dideoxy-9 α ,11 α -methanoepoxy-prostaglandin F_{2 α} (U46619). The length of the RbAs was monitored with auxotonic levers attached to a Watanabe WR3101 recorder. Glyceryl trinitrate (GTN) was used to calibrate the response of the detector tissues which were electronically adjusted to give similar relaxations to a standard dose. EDRF release from endothelial cells is characterized by its progressive disappearance down the cascade. The intracellular concentration of L-Arg, L-Gln and other amino acids was determined by reversed phase high performance liquid chromatography/fluorescence detection

analysis as previously described (Hecker *et al.*, 1990). Adenosine diphosphate di(monocyclohexylammonium) salt (ADP), the amino acids and indomethacin were obtained from Sigma Chemical Co. (Poole, U.K.). Superoxide dismutase from bovine erythrocytes was a gift from Grünenthal (Stolberg, West Germany). GTN (Nitronal) was supplied by Lipha Pharmaceuticals Ltd (West Drayton, U.K.).

Results The effect of L-Gln on the release of EDRF from endothelial cells cultured in the presence of L-Arg is shown in Figure 1. L-Gln (0.2 mM and 2 mM) had no effect on the detector tissues but significantly reduced the release of EDRF induced by ADP ($46.5 \pm 5.5\%$ inhibition ($n = 8$) and $48.1 \pm 7.2\%$ inhibition ($n = 3$); mean $\pm$ s.e.mean; $P < 0.05$) when infused through the column of endothelial cells. This inhibition was overcome by co-infusions of 100 μ M L-Arg ($83.6 \pm 5.7\%$ of control ($n = 6$) and $106.0 \pm 2.7\%$ of control ($n = 3$); $P < 0.05$) but not D-Arg ($53.5 \pm 9.9\%$ of control in the presence of 200 μ M L-Gln, ($n = 3$)).

Infusions of L-Gln (2 mM) similarly inhibited the release of EDRF from Arg-depleted endothelial cells (Figure 2). As described previously (Mitchell *et al.*, 1990a), there was a time-dependent increase in stimulated EDRF release over 60 min which was prevented when L-Gln (0.2 mM or 2 mM) was present from the beginning of the experiment ($n = 3$). The release of EDRF induced by ADP (6 nmol) was attenuated by L-Gln in concentrations as low as 20 μ M ($31.1 \pm 2.3\%$ inhibition ($n = 3$); $P < 0.05$) and maximally inhibited at 200 μ M ($52.5 \pm 4.5\%$ inhibition ($n = 3$) as compared to $53.2 \pm 4.3\%$ inhibition ($n = 4$) at 2 mM). L-Arg (50 μ M) but not D-Arg reversed the inhibitory effect of 2 mM L-Gln ($94.6 \pm 10.2\%$ of control, ($n = 4$)). D-Gln (0.2 mM and 2 mM), L-glutamic acid (0.2 mM and 2 mM) or ammonium chloride (1 mM) had no effect on EDRF release ($n = 4$).

When transferred to Krebs solution for 60 min at 37°C, endothelial cells cultured in the absence or presence of L-Arg increased their L-Arg levels by 9.5 ± 2.0 fold from $34 \pm 4 \mu$ M to $210 \pm 24 \mu$ M ($n = 26$) and 4.5 ± 1.1 fold from $160 \pm 21 \mu$ M to $641 \pm 125 \mu$ M ($n = 6$), respectively. L-Gln (0.2 mM and 2 mM) significantly inhibited the rise in intracellular L-Arg in both Arg-depleted ($81.5 \pm 6.3\%$ inhibition ($n = 5$) and $79.7 \pm 10.0\%$ inhibition ($n = 6$); $P < 0.05$) and non-depleted cells ($95.9 \pm 2.1\%$ inhibition at 0.2 mM ($n = 4$); $P < 0.05$). D-Gln (2 mM) potentiated (2.4 ± 0.7 fold), ($n = 3$) rather than inhibited the generation of L-Arg, whereas L-glutamic acid (2 mM) and ammonium chloride (1 mM) were not active ($16.5 \pm 7.2\%$ inhibition and $14.1 \pm 7.9\%$ inhibition), ($n = 4$).

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Figure 1 The effect of L-glutamine (L-Gln) on the release of endothelium-derived relaxing factor (EDRF) from endothelial cells cultured in the presence of L-arginine (L-Arg). Of four rabbit aortic strips (RbA) two are shown precontracted with 30 mM U46619 and separated by 1 s and 7 s from the column of endothelial cells. Adenosine diphosphate (ADP, 6 nmol) injected through the column of endothelial cells (t.c.) elicited a release of EDRF which caused a greater relaxation of the top RbA than glyceryl trinitrate (GTN, 40 pmol) given over the tissues (o.t.) L-Gln (2 mm) infused t.c. caused an increase in the tone of the RbAs and a reduction in stimulated EDRF release (the relaxation of the top RbA was smaller as compared to GTN). These effects were reversed by a co-infusion of L-Arg (100 μ M). Relaxations to GTN (40 pmol) given o.t. were not affected.

Discussion Endothelial cells cultured in the absence of L-Arg ($[Arg]_i < 10 \mu$ M) generate L-Arg ($[Arg]_i > 100 \mu$ M) when transferred to Krebs solution and the availability of L-Arg appears to be rate-limiting for EDRF release (Mitchell *et al.*, 1990a). As the generation of L-Arg in these cells coincided with a rapid fall in L-Gln (Mitchell *et al.*, 1990b), probably due to

their high glutaminase activity (Leighton *et al.*, 1987), and since L-Gln was a major constituent of the culture medium (2 mm), it was tempting to speculate that L-Gln may block the generation of L-Arg and, hence, EDRF biosynthesis. Indeed, L-Gln but not D-Gln inhibited the release of EDRF from both Arg-depleted and non-depleted endothelial cells. L-Gln also

Figure 2 The effect of L-glutamine (L-Gln) on the release of endothelium-derived relaxing factor (EDRF) from endothelial cells cultured in the absence of L-arginine (L-Arg) for 24 h. Two of four rabbit aortic strips (RbA) are shown separated as in Figure 1. Repetitive injections of adenosine diphosphate (ADP, 6 nmol) through the column of endothelial cells (t.c.) elicited a release of EDRF which increased over 60 min. L-Gln (2 mm) infused t.c. reduced the release of EDRF induced by ADP. This effect was reversed by a subsequent co-infusion of L-Arg (50 μ M). Relaxations induced by glyceryl trinitrate (GTN) given over the tissues (o.t.) were not affected.

prevented the time-dependent increase in stimulated EDRF release from Arg-depleted endothelial cells. This process is probably linked to the generation of L-Arg (Mitchell *et al.*, 1990a), and, consequently, the effect of L-Gln on the generation of L-Arg was investigated. L-Gln but not D-Gln, L-glutamic acid or ammonium chloride inhibited the generation of L-Arg by both Arg-depleted and non-depleted endothelial cells in the same concentration range as in the bioassay experiments, strongly suggesting that L-Gln, indeed, inhibits EDRF biosynthesis by blocking the generation of L-Arg.

L-Gln also stereospecifically inhibited the release of EDRF from non-depleted endothelial cells, supporting the notion that these cells maintain their L-Arg level and, hence, sustain their ability to release EDRF by generating L-Arg possibly from L-citrulline, the by-product of EDRF biosynthesis (Mitchell *et al.*, 1990b). The glutaminase metabolites, L-glutamic acid and ammonia, had no effect on either EDRF release or L-Arg generation. Although the precise mechanism

by which L-Gln interferes with the generation of L-Arg remains to be elucidated, this finding together with the lack of inhibitory effect of D-Gln indicates a direct interaction of L-Gln with the enzyme(s) involved in L-Arg generation.

L-Gln is the most abundant amino acid in the body (Bulus *et al.*, 1989), its concentrations is 0.5–0.9 mM in plasma and about 2 mM in tissues or cells, including endothelial cells (Mitchell *et al.*, 1990b). The inhibition of EDRF release by L-Gln *in vitro* was maximal at 200 μ M, a concentration well within the physiological range. Thus, L-Gln may be an endogenous inhibitor of EDRF biosynthesis *in vivo* when the plasma level of L-Arg is low or when the uptake of L-Arg by the endothelium is limited.

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