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The role of nitric oxide in endothelial cell damage and its inhibition by glucocorticoids

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Incubation of vascular endothelial cells with *S.typhosa* endotoxin and interferon-γ caused a time- and concentration-dependent reduction in the viability of the cells. This cytotoxic effect was inhibited in a concentration-dependent manner by N^G-monomethyl-L-arginine, an inhibitor of nitric oxide (NO) synthesis, and by the glucocorticoids dexamethasone and hydrocortisone, two inhibitors of the induction of NO synthase. These findings indicate that in these cells the cytotoxic effect of endotoxin is mediated by the NO synthesized by an inducible NO synthase. This induction of NO synthase in vascular endothelial cells may represent a mechanism of local endothelial damage during endotoxin shock and other immunologically based conditions.

Keywords: Endothelium; nitric oxide synthase; cytotoxicity; glucocorticoids; endotoxin

Introduction Vascular endothelial cells synthesize nitric oxide (NO) from L-arginine by the action of a Ca^{2+}-dependent constitutive NO synthase as a transduction mechanism for the activation of the soluble guanylate cyclase in vascular smooth muscle. The consequent rise in guanosine 3':5'-cyclic monophosphate (cyclic GMP) causes vascular relaxation which contributes to the regulation of blood pressure in animals and man (for review see Moncada *et al.*, 1991).

Nitric oxide also plays a role in the cytotoxic activity of macrophages (for review see Hibbs *et al.*, 1990). These cells synthesize NO by a Ca^{2+}-independent enzyme that is induced following activation by endotoxin (LPS) and/or cytokines (Marletta *et al.*, 1988). Induction of this enzyme also occurs in other cells, including vascular endothelium (Radomski *et al.*, 1990). The vascular endothelial cells are the only cells so far known to possess both the constitutive and inducible NO synthases. The functional consequences of the formation of NO by the inducible NO synthase have not been clearly established, but it is likely that, besides cytotoxicity for micro-organisms and tumour cells, it plays a role in pathological vasodilatation and tissue damage (for review see Moncada *et al.*, 1991). The expression, but not the activity, of the inducible NO synthase is inhibited by glucocorticoids such as dexamethasone and hydrocortisone. This action is distinct from that of arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) which are specific inhibitors of both the constitutive and inducible NO synthases.

Using these two types of inhibitors we have now examined whether endothelial cells are damaged by the NO synthesized as a consequence of the expression of the inducible NO synthase.

Methods Vascular endothelial cells were isolated as described previously (Gryglewski *et al.*, 1986) by collagenase digestion from porcine aortae obtained from a local abattoir. The cells were cultured at 37°C for 48 h in Dulbecco's modification of Eagle's medium (400 μM L-arginine) containing 20% foetal calf serum, penicillin ($100\,u\,ml^{-1}$) and streptomycin ($100\,\mu g\,ml^{-1}$). The cultures were then screened microscopically for microbial contamination, before being subcultured until confluent (72 h) in 6-well plates as described above (approximately 1×10^6 cells per well).

When confluent, the cells were incubated for the appropriate time (generally 24 h) with LPS and/or interferon-γ (IFN-γ) and, where indicated, with test compounds. After incubation, the culture supernatant was removed and replaced with 0.5% trypan blue in phosphate buffered saline and incubation continued for a further 10 min at 37°C. The number of dead cells was then determined, by light microscopy at ×200 magnification, by counting those cells that failed to exclude the dye in each of three representative fields in each of three replicate wells. Statistical significance was determined by Student's paired t test and $P < 0.05$ taken as significant.

Results Incubation of vascular endothelial cells with LPS ($10\,\mu g\,ml^{-1}$) or IFN-γ ($150\,u\,ml^{-1}$) for 24 h caused concentration-dependent cytotoxicity, as measured by exclusion of trypan blue. LPS alone was significantly more effective than IFN-γ (Figure 1a). The maximum effect observed with LPS ($100\,\mu g\,ml^{-1}$) was 137.4 ± 9.1 as compared with 52.0 ± 1.9 dead cells per field ($n = 4$ for each) for IFN-γ ($1500\,u\,ml^{-1}$). These represented 22.9 ± 1.9 and 8.7 ± 0.3% of the total number of cells in each field respectively. Concomitant incubation of the cells with threshold concentrations of these agents for 24 h did not lead to a synergistic interaction; LPS $0.1\,\mu g\,ml^{-1}$, IFN-γ, $15\,u\,ml^{-1}$ and LPS $0.1\,\mu g\,ml^{-1}$ with IFN-γ $15\,u\,ml^{-1}$ caused 21.8 ± 6.8, 8.8 ± 1.6 and 32.1 ± 4.9 dead cells per field respectively, $n = 3$ for each.

Incubation of the cells with LPS ($10\,\mu g\,ml^{-1}$) and IFN-γ ($150\,u\,ml^{-1}$) caused time-dependent cytotoxicity which was significant at 6 h and reached a maximum at 24 h (Figure 1b). This level was not significantly increased by incubation of the cells for a further 24 h either in the same medium (Figure 1b) or in fresh medium containing LPS ($10\,\mu g\,ml^{-1}$) and IFN-γ ($150\,u\,ml^{-1}$; $n = 3$).

Cytotoxicity in arginine-containing (400 μM) medium was inhibited in a concentration-dependent manner by L-NMMA (Figure 2), but not by D-NMMA (100 μM) or by 10 μM haemoglobin ($n = 3$ for each). Hydrocortisone and dexamethasone also caused concentration-dependent inhibition of cytotoxicity under these conditions (Figure 2). The cytotoxic effect of incubation with LPS ($10\,\mu g\,ml^{-1}$) and IFN-γ ($150\,u\,ml^{-1}$) for 24 h was reduced by 40.8 ± 5% when the cells were cultured in L-arginine-free medium. In contrast, indomethacin (5 μM) significantly potentiated the cytotoxic effect of these concentrations of LPS and IFN-γ from 68.0 ± 6.6 to 129.5 ± 7.9 dead cells/field.

Discussion LPS and IFN-γ, which induce the expression of a Ca^{2+}-independent NO synthase in vascular endothelial cells (Radomski *et al.*, 1990), caused a time-dependent decrease in viability, which became apparent after a lag period of 6 h and was inhibited by L-NMMA but not D-NMMA. These findings indicate that this cytotoxicity is mediated by NO synthesized by an inducible enzyme. A similar effect associated with the induction of NO synthesis has recently been described in

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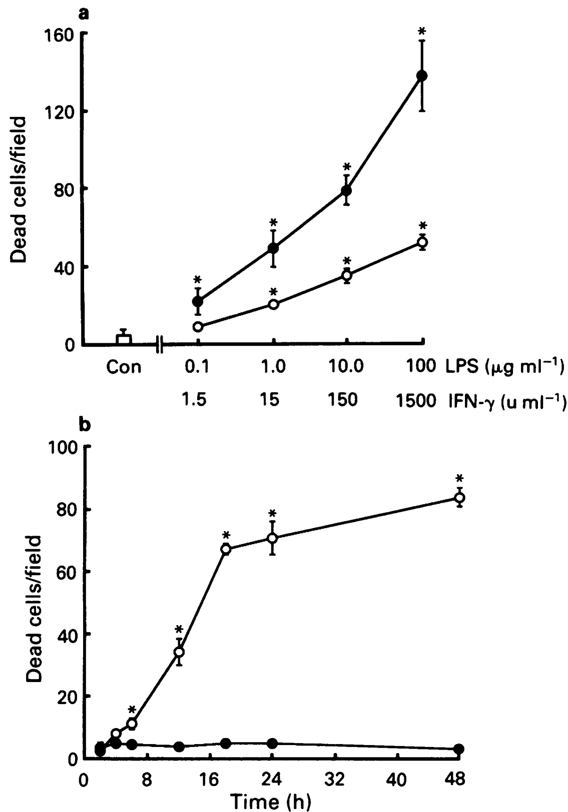

Figure 1 (a) Effect of endotoxin (LPS, ●) and interferon-γ (IFN-γ, ○) on the viability of endothelial cells. Each point is the mean of three or four separate experiments; s.e.mean shown by vertical bars. Con = medium alone. (b) Time-dependent development of cytotoxicity (○) during incubation of endothelial cells with LPS ($10\,\mu g\,ml^{-1}$) and IFN-γ ($150\,u\,ml^{-1}$); (●) medium alone. * $P < 0.05$ compared to corresponding time control.

EMT-6 cells (O'Connor & Moncada, 1991). Interestingly, the cytotoxic effect of NO was not affected by haemoglobin, suggesting that the NO synthesized exerts its cytotoxic effect directly without being released into the extracellular space.

The development of this NO-mediated cytotoxicity was also inhibited by the glucocorticoids dexamethasone and hydrocortisone, which prevent the induction of NO synthase in several tissues *in vitro* and *in vivo* (see Moncada *et al.*, 1991). In addition, the cytotoxic effect of LPS and IFN-γ was attenuated in the absence of L-arginine. These findings provide further evidence for an NO-dependent cytotoxicity in endothelial cells. Since cytotoxicity was only partially reduced in the absence of L-arginine, it is likely that endothelial cells contain sufficient L-arginine to synthesize substantial amounts

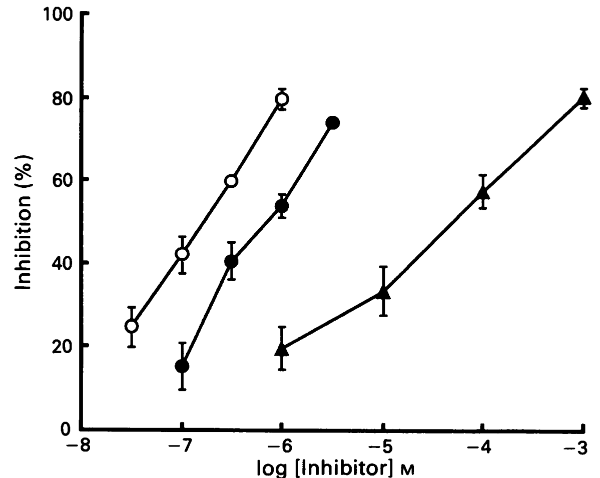

Figure 2 Effect of dexamethasone (○), hydrocortisone (●) and N^G-monomethyl-L-arginine (L-NMMA, ▲) on the cytotoxicity induced in endothelial cells by endotoxin (LPS, $10\,\mu g\,ml^{-1}$) and interferon-γ (IFN-γ, $150\,u\,ml^{-1}$). Each point is the mean of four separate experiments; vertical bars show s.e.mean.

of NO or that they are able to synthesize L-arginine from other cellular components (Hecker *et al.*, 1990).

The cytotoxic effect of LPS and IFN-γ reached a maximum at 24 h and affected less than 25% of the cells. There was no additional increase in cytotoxicity following a further 24 h incubation in fresh medium containing these agents. The reason for this limited cytotoxicity requires further investigation.

The cytotoxic effect of LPS and IFN-γ was significantly enhanced by concomitant incubation with indomethacin. The mechanism by which eicosanoid synthesis affects NO-mediated cytotoxicity is not known but it may either be due to a cytoprotective effect of prostacyclin (Blackwell *et al.*, 1982) or to a down-regulation of the induction of NO synthase. Interestingly cyclo-oxygenase has recently been reported to be induced in endothelial cells by LPS and this induction is inhibited by glucocorticoids (Masferrer *et al.*, 1990). This suggests that during immunological activation these cells are able to release cytoprotective and cytotoxic substances, the balance of which may determine the final outcome in terms of host tissue viability.

Our finding that endothelial cells are damaged as a consequence of the induction of NO synthase provides an explanation for the local endothelial damage that can occur in immunologically-based conditions. That the vascular endothelium releases NO constitutively for physiological purposes and also expresses NO synthesis as part of the host defence mechanism exemplifies the diverse roles of NO as a biologically active molecule.

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