Cells and in response to external stimuli such as growth.

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Polyamines, arginine and nitric oxide

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Putrescine [NH₂(CH₂)₂NH₂], spermidine [NH₂(CH₂)₁NH(CH₂)₁NH] and spermine [NH₂(CH₂)₁-NH(CH₂)₁NH(CH₂)₁NH] are normal cellular constituents essential for cell growth and differentiation [1–3]. Their concentrations vary with the cell cycle, and in response to external stimuli such as growth factors and hormones. Induction of polyamine biosynthesis from l-ornithine is one of the first events in cell proliferation. Intracellular polyamine concentrations are controlled by a tightly regulated synthetic pathway and also by a series of catabolic reactions.

Polyamine transport

Many cell types [4,5], including vascular endothelial cells [6,7], possess an uptake system for polyamines that is distinct from those for the amino acids and which, in certain circumstances, can substitute for synthesis de novo. In some cells, polyamines are taken up by both saturable and non-saturable systems. Uptake by saturable systems is energy-requiring, temperature-dependent and carrier-mediated and operates against a substantial concentration gradient [8,9]. In many cells, putrescine and, often, spermidine transport is Na⁺-sensitive (the term Na⁺-sensitive is preferable to the more frequently used Na⁺-dependent, as removal of extracellular Na⁺ reduces, but often does not abolish, uptake). In contrast, spermine transport in most cells is not affected by changes in extracellular Na⁺ concentrations.

Transport can be inhibited by reagents, such as N-ethylmaleimide or p-chloromercuribenzoate, that alkylate sulphhydryl groups, and stimulated by sulphhydryl donors such as dithiothreitol, suggesting a requirement for SH groups on the transporter(s). Frequently, N-ethylmaleimide, which can be taken up by cells, is found to be a more effective inhibitor
than the less permeant p-chloromercuribenzoate, suggesting that the SH groups involved in polyamine transport are located within, rather than on, the cell membrane. The number of carriers in the polyamine transport system varies with cell type. Human umbilical vein endothelial cells contain apparently two carriers, one shared by spermine and spermidine, and one capable of transporting all three polyamines [6]. In contrast, porcine aortic endothelial cells appear to possess three carriers, one for each polyamine [7]. Multiple carriers are not uncommon although many cell types appear to possess a single polyamine carrier [4].

**Regulation of polyamine transport**

The mechanisms of polyamine transport and the means by which polyamine uptake is induced are not clear. Uptake is generally low in quiescent cells, or in cells that have been induced to differentiate, in contrast to cells undergoing rapid growth, where uptake is enhanced. Uptake is also increased in response to proliferative stimuli such as serum, growth factors and hormones, and in cells in which polyamine stores have been depleted, for example by the use of inhibitors of polyamine biosynthesis such as difluoromethylornithine [7,10–12], an enzyme-activated inhibitor of ornithine decarboxylase [13]. Putrescine and spermine uptake by Chinese hamster ovary (CHO) cells was inhibited by treatment with cycloheximide (10 µg/ml) for 4–24 h [14]. Putrescine uptake was also reduced in mouse hepatocytes cultured in the presence of 20 µM cycloheximide for 20 h, and treatment with 100 µM actinomycin D for the same period virtually abolished uptake; in both cases, viability was unaffected [15]. These data suggest that uptake, in these cells, was dependent on the synthesis of both new protein and new mRNA. In contrast, brief exposure of cells from the human umbilical vein endothelial cell line ECV304 [16] to cycloheximide (0.1 mM, 15 min) resulted in a marked increase in the uptake of putrescine and spermidine (2–3-fold above control values), and treatment with actinomycin D (5 µg/ml) for the same period also increased uptake but to a lesser extent (25–50% above control values; D. M. L. Morgan and O. Rajanayagam, unpublished work). Treatment of cultures of rat hepatoma (IITC) cells for 3–6 h with puromycin (200 µg/ml) or cycloheximide (0.2 mM) in the presence of 50 µM spermidine resulted in sustained uptake of exogenous spermidine to intracellular levels 1.5–3-fold greater than in control cells [17]. These findings have been interpreted as suggesting that one factor in the regulation of polyamine uptake may be the rapid, reversible inactivation of a polyamine carrier by a short-lived protein, synthesis of which is stimulated by a rise in intracellular polyamine levels. The apparent contradiction between these two sets of results may be a consequence of the differences in time of exposure and concentration of the inhibitors. It could be argued that the major effect of short exposure times would be to deplete the cell of proteins which turn over rapidly. Prolonged exposure would result also in the depletion of proteins with longer half-lives. Thus these data could be interpreted as indicating that polyamine transport is regulated by both a rapidly degraded protein inhibitor, which responds to a rise in intracellular polyamine levels, and a longer-lived protein, or proteins, which may be the polyamine carrier(s).

**Precursors for polyamine biosynthesis**

The precursor for polyamine biosynthesis in mammalian cells is ornithine, and the commonly accepted source of this amino acid is the plasma. The ornithine concentration in human plasma is ~85 µM [18] (the plasma arginine concentration is ~90 µM); foetal calf serum, a major component in most tissue-culture media, contains much higher levels of ornithine (~300 µM) and less arginine (40–50 µM) [19]. However, many cell types, including vascular endothelial cells (D. M. L. Morgan and P. Rezaie, unpublished work), contain an arginase, even when lacking a complete urea cycle [20]. In these cells, the ornithine required for polyamine synthesis may be derived from arginine. Evidence of links between arginine and polyamines comes from a number of sources. Growth of an arginase-deficient CHO cell variant was reduced when cultured in a medium lacking ornithine, or polyamines, or both [21]. Diminished epidermal tumourigenesis was observed in mice maintained on an arginine-free diet and this reduction correlated with a 40% reduction in epidermal ornithine and arginine levels [22]. Putrescine levels in the livers of arginine-starved rats decreased by 50% within 8 days and were restored to control values upon refeeding [23]. Culture of porcine aortic endothelial cells in arginine-free medium resulted in a marked and rapid depletion in intracellular arginine to 50% of control values [24]. This reduction in arginine pool size was accompanied by a significant increase in putrescine [7] and spermidine transport; spermine uptake was not increased under these conditions (D. M. L. Morgan, unpublished work). Supplementation of the arginine-free medium with 0.1 mM ornithine reduced, but did not abolish, the increase in putre-
Nitric oxide

Nitric oxide (NO) is now recognized as an important transcellular signal, regulating not only blood-vessel dilatation and immune function, but also implicated in host defence mechanisms and neurotransmission [35]. NO is also a mediator of both the cytotoxic and cytostatic actions of macrophages [36].

Macrophages or macrophage/monocyte cell lines are widely used in NO studies as they express high levels of NO synthase and produce large amounts of NO in response to suitable stimuli. The expression of NO synthase reaches a peak 12 h after stimulation and declines to resting levels after ~72 h. Cells can be reactivated several times and reactivation is accompanied by re-expression of NO synthase mRNA [38]. Macrophages are recognized as the target cells of a number of substances known to act as immunoadjuvants. These include cell walls from mycobacteria (commonly Mycobacterium bovis strain Bacillus Calmette-Guerine; BCG) and lipopolysaccharide (LPS) from Escherichia coli [39,40].

Studies of bacterial cell-wall structure led to the synthesis of the glycopeptide N-acetylmuramyl-L-alanyl-D-isoglutamine (muramylidipeptide), which represents the minimal chemical structure required for adjuvant activity [41–43]. Treatment with E. coli LPS [44], BCG [45] or muramylidipeptide [46] can induce synthesis of NO in macrophages and in murine macrophage cell lines. Release of NO, usually measured as nitrite, from stimulated cells is both dose- and time-dependent. Detectable amounts of nitrite appeared in the medium of cultures of macrophages exposed to LPS (0.5–1 μg/ml) after ~6 h and release continued for 24–36 h, but was blocked by the addition of cycloheximide (10 μg/ml) [47].

NO synthase converts arginine to NO with citrulline as the other reaction product. The constitutively present isoform of the enzyme in endothelial cells is Ca^{2+}-/calmodulin-sensitive, and releases NO for short periods in response to receptor or physical stimulation. In contrast, the synthesis of a Ca^{2+}-insensitive isoform, absent from unstimulated cells, is induced in macrophages, endothelial and other cells in response to cytokines, and synthesizes NO for long periods [37]. Thus, in NO-secreting cells, there are potentially two arginine-requiring pathways. Are there links between these two pathways? A growing body of evidence suggests that there are.

Polyamines and NO

Exposure to BCG or LPS was found to increase ornithine decarboxylase (ODC) activity in macrophages [48]. Both of these immunoadjuvants, and muramylidipeptide, induced increases in ODC activity and protein synthesis in the macrophage cell lines, PUS-1.8 and 7774. ODC activity increased ~2 h after adjuvant stimulation, reached a peak at 4 h and had returned to control levels by ~8 h. The increase in protein synthesis, which also was maximal at 4 h was, however, sustained for at least 24 h [49]. Thus the increase in ODC activity preceded nitrite release, which was temporally related to the increase in protein synthesis. Cells of the murine macrophage-leukemic cell line RAW 264 constitutively secrete putrescine into the culture medium at a significant rate. Secretion was increased by >50% following addition of LPS (1 μg/ml, 25 h), and intracellular putrescine increased 3-fold [50]. Ornithine (1 mM) produced a similar increase in putrescine export without a concomitant rise in intracellular levels; LPS and ornithine in combination resulted in a further increase in putrescine secretion, and also elevated intracellular putrescine...
5-fold. Spermine and spermidine levels, both intracellular and extracellular, were unaffected. LPS treatment also increased ODC activity in these cells; activity reached a peak at 4 h, had decreased by 50% below the maximum at 6 h but was still 4-fold greater than control levels at 8 h. LPS treatment also stimulated nitrite release, and this was not enhanced by the concomitant addition of ornithine. Interferon-γ (10 i.u./ml), another macrophage activator, was about equipotent in stimulating nitrite release but, when combined with LPS, synergistically increased nitrite release 3-fold more than was achieved by either compound alone. Interestingly, addition of ornithine (1 mM) to this combination resulted in a further stimulation of nitrite production [50].

Treatment of J774 cells with DFMO (5 mM) for 24 h prior to activation with LPS inhibited nitrite production in a time- and dose-dependent manner [51]. Inhibition of polyamine biosynthesis might be expected to increase nitrite production by blocking one of the arginine-requiring pathways, so making more arginine available for the synthesis of nitrite. However, in macrophages, synthesis of NO by the inducible NO synthase in response to agents such as LPS is critically dependent on extracellular arginine [52], and ornithine has been shown to inhibit arginine transport [53]. Hence the decrease in nitrite production might result from DFMO emulating ornithine and inhibiting arginine uptake. However, substitution of DFMO with equimolar concentrations of ornithine neither enhanced or diminished LPS-stimulated production by J774 cells.

This observation negates the possibility that, by blocking ODC, DFMO increased ornithine accumulation which, in turn, inhibited arginine entry into the cells. The only known effect of DFMO, to date, is inhibition of ODC [13]. If, in these cells, ornithine is derived from arginine then there may be an accumulation of ornithine or arginine but this is more likely to be stimulatory rather than inhibitory. Inhibition of NO production by DFMO was progressive, suggesting that it may be irreversible. DFMO may act directly on NO synthase to block conversion of arginine to NO. Alternatively, the presence of reactive fluorine atoms in DFMO may result in tight, and perhaps irreversible, binding to the arginine transporter. If DFMO acts by inhibiting arginine transport, then it is a more potent inhibitor than ornithine [53]. A more probable explanation is that DFMO inhibition of nitrite production by the inducible NO synthase may be indirect. As noted above, nitrite release in response to LPS is preceded by protein synthesis which, in turn, is preceded by a rise in ODC activity. By preventing this increase in ODC activity, DFMO may have a downstream effect on nitrite synthesis and release.

Recently it has been reported that spermine (10 μM–1 mM) inhibited nitrite production by LPS-treated J774 cells, but only in the presence of foetal calf serum [54]. This raises the possibility that the active agent is not the polyamine but the mono- or dialdehyde product resulting from oxidation of spermine by bovine serum amine oxidase [2,55]. Repetition of the experiment in the presence of an inhibitor of the oxidase would help to resolve this point. However, it should be born in mind that aminoguanidine, an inhibitor of bovine serum amine oxidase [55], is also an effective inhibitor of the inducible NO synthase [56,57].

Another, and more speculative, area where therapeutic intervention in either polyamine metabolism or NO production may have unexpected side-effects is their effects on the N-methyl-L-aspartate (NMDA) subtype of excitatory amino acid receptors of the central nervous system. Spermine and spermidine interact with a specific polyamine recognition site on the NMDA receptor to modulate the binding of open-channel blockers, such as phenylcyclidine, to the receptor complex [58]. NO can also inhibit responses mediated by the NMDA receptor, possibly by reacting with free sulphydryl groups on the receptor-channel complex [59].

Arginine can be a precursor for both polyamine and NO synthesis. The data reviewed here suggest that clinical manipulation of one of these arginine-requiring pathways may have unforeseen effects on the other.

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Polyamines in Clinical and Basic Science


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