bility that brain barrier tissues may be end organs for atriopeptins, findings that are relevant to CNS water regulation under normal conditions and in pathophysiological situations such as cerebral edema or hydrocephalus. Our findings also suggest that isolated choroid epithelial cells may provide a useful model system for studying the receptor and post-receptor actions of atriopeptins in a defined cell population. For example, characterization of tertiary messengers, such as proteins whose phosphorylation is regulated by cyclic GMP–dependent protein kinase, should provide clues to biochemical mechanisms mediating the physiological effects of atriopeptins.

Note added in proof: In recent autoradiographic studies, McCarthy and Plunkett (*13*) have reported that labeled rANP binds to whole rat choroid plexus with an affinity (7 n*M*) similar to that which we found for activation of guanylate cyclase in rabbit choroid plexus.

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Macrophage Cytotoxicity: Role for L-Arginine Deiminase and Imino Nitrogen Oxidation to Nitrite

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Previous studies have shown that cytotoxic activated macrophages cause inhibition of DNA synthesis, of mitochondrial respiration, and of aconitase activity in tumor target cells. An L-arginine–dependent biochemical pathway synthesizing L-citrulline and nitrite, coupled to an effector mechanism, is now shown to cause this pattern of metabolic inhibition. Murine cytotoxic activated macrophages synthesize L-citrulline and nitrite in the presence of L-arginine but not D-arginine. L-Citrulline and nitrite biosynthesis by cytotoxic activated macrophages is inhibited by N^G-monomethyl-L-arginine, which also inhibits this cytotoxic effector mechanism. This activated macrophage cytotoxic effector system is associated with L-arginine deiminase activity, and the imino nitrogen removed from the guanido group of L-arginine by the deiminase reaction subsequently undergoes oxidation to nitrite. L-Homoarginine, an alternative substrate for this deiminase, is converted to L-homocitrulline with concurrent nitrite synthesis and similar biologic effects.

MOUSE PERITONEAL MACROphages activated in vivo by intracellular pathogens such as *Mycobacterium bovis*, strain Bacillus Calmette-Guérin (BCG), or in vitro by lymphokines, are cytotoxic for tumor cells after exposure to a second signal such as lipopolysaccharide (LPS) (*1–4*). Cytotoxic activated macrophages (CAM) cause a slowly developing and reproducible pattern of metabolic inhibition in tumor target cells which, in many cases, is fully reversible. For example, CAM cells inhibit mitochondrial respiration (*5–7*), the citric acid cycle enzyme aconitase (*7*), and DNA synthesis (*8*), while certain other metabolic pathways such as glycolysis remain functional (*5*). This reproducible and selective pattern of metabolic inhibition is, at least in part, due to CAM-induced iron loss from tumor target cells (*7, 9*). Earlier experiments showed that CAM-induced metabolic inhibition in tumor target cells is L-arginine–dependent (*10*). We therefore tested L-arginine and its homolog L-homoarginine, which substitutes for L-arginine as an inducer of CAM-associated cytotoxicity (*10*), as substrates for a CAM-associated deiminase synthesizing L-citrulline and L-homocitrulline, respectively. Arginine deiminase activity has not been described previously in mammalian cells (*11*).

L-Citrulline accumulation in the medium was measured after 24 hours by a colorimetric reaction that detects carbamido compounds (*12*). Urea was removed from samples prior to the assay by incubation with urease (1 unit/ml) at 37°C for 1 hour. The experiments were performed in medium prepared without amino acids except L-glutamine (*13*) and the desired concentration of L-arginine, L-homoarginine, or D-arginine. L-Glutamine does not affect the L-arginine–dependent CAM-effector mechanism (*10*). In the presence of L-arginine but not D-arginine, CAM cultured alone and CAM cocultivated with L10 cells released a molecule containing the carbamido group (L-citrulline) into the medium (Table 1). The quantity of L-citrulline synthesized was proportional to the initial L-arginine concentration. Table 1 also shows a correlation between the L-arginine concentration in the medium, the synthesis of L-citrulline, and the development of inhibition of mitochondrial respiration in L10 target cells as determined by lysis in a glucose-free second incubation medium. These results demonstrate an L-arginine–dependent CAM effector mechanism with deiminase activity that converts L-arginine to L-citrulline. L-Homoarginine is also a substrate for this deiminase and when added to the medium under identical conditions of culture or coculture produced similar results (Table 1).

$$\underset{\text{(L-homoarginine)}}{\text{L-arginine}} + H_2O \xrightarrow{\text{Deiminase}} \underset{\text{(L-homocitrulline)}}{\text{L-citrulline}} + NH_3$$

$$NH_3 + 1\text{-}1/2\ O_2 \xrightarrow{\text{Oxidase}} NO_2^- + H_2O + H^+$$

Fig. 1. Proposed pathway for L-citrulline and L-homocitrulline synthesis from L-arginine and L-homoarginine with subsequent oxidation of imino nitrogen to nitrite.

Arginine deiminase converts L-arginine to L-citrulline and ammonia (Fig. 1). We used an experimental protocol identical to that outlined in Table 2 to measure ammonia in the culture medium. Ammonia was measured by two methods—ion exchange chromatography (Biotronik LC 5001) and an enzymatic assay (*14*) (Aca II clinical analyzer, DuPont). We found no correlation between the ammonia concentration in the

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medium and development of CAM-induced metabolic changes in target cells.

Stuehr and Marletta (*15*) reported that murine macrophages exposed to LPS synthesize nitrite and nitrate. Using the Griess reaction (*15*), which is a sensitive technique for measuring nitrite but which does not detect nitrate, we found evidence that imino nitrogen removed from L-arginine by the deiminase reaction is oxidized to nitrite and that nitrite production also is linked to expression of CAM-induced metabolic changes in target cells. The pattern of nitrite synthesis was similar to that found for L-citrulline synthesis (Table 1). The amount of nitrate measured was proportional to the initial L-arginine concentration and, as with L-citrulline, the increase correlated with inhibition of mitochondrial respiration in L10 cells. Supplementing the medium with D-arginine did not result in increased nitrite synthesis. Since nitrite constitutes ~60% of the total macrophage nitrite and nitrate production (*15*), the concentration of nitrite we measured does not detect all the imino nitrogen actually oxidized to nitrite and nitrate.

The culture medium used in the experiments described so far contained L-glutamine as well as L-arginine or L-homoarginine. To determine whether L-glutamine–associated nitrogen contributes to nitrite synthesis by CAM, we measured nitrite after a 24-hour cultivation in medium containing 1.2 m*M* L-arginine plus 4 m*M* L-glutamine (96 ± 5 μ*M* nitrite synthesized; average of six experiments ± SEM) and in medium containing 1.2 m*M* L-arginine alone (97 ± 3.5 μ*M* nitrite synthesized; average of six experiments ± SEM). This shows that L-glutamine–associated nitrogen does not contribute to nitrite synthesis under the conditions of our in vitro assay.

There is a small amount of citrulline and nitrite synthesis by CAM cultured without L-arginine (Table 1). This baseline synthesis is lower if CAM cells are cultured in medium without serum. This suggests that L-arginine is produced from endocytosed serum proteins as well as from endogenous sources. The data indicate that the activity of the enzyme or enzymes constituting the effector system exceeds a threshold rate for development of inhibition of mitochondrial respiration in L10 target cells only in the presence of exogenous L-arginine.

Next we examined the possibility that CAM-induced inhibition of aconitase and DNA synthesis in L10 target cells is linked to L-arginine deiminase activity and oxidation of imino nitrogen to nitrite. L-Citrulline in the medium was identified and quantitated by ion exchange chromatography (Biotronik LC 5001 amino acid analyzer). Table 2 shows that increased amounts of L-citrulline and nitrite were synthesized by CAM after 7.5 hours of coculture in medium containing L-arginine. As the incubation time was lengthened to 10 hours and 24 hours, the concentration of L-citrulline and nitrite in the medium continued to increase. When the medium lacked L-arginine, accumulation of L-citrulline was not detectable by ion exchange chromatography, which is specific but less sensitive than the colorimetric method. N^G-monomethyl-L-arginine (N^GMMA), an inhibitor of the L-arginine–dependent CAM effector system (*10*), also prevented or decreased the production of L-citrulline and nitrite. In addition, Table 2 shows that the development of CAM-dependent inhibition of aconitase and DNA synthesis in L10 target cells was correlated with increased biosynthesis of L-citrulline and nitrite. CAM-induced inhibition of aconitase and DNA synthesis in L10 cells did not occur when the medium lacked L-arginine or when N^GMMA was present. When N^GMMA alone was added to the medium, nitrite synthesis from endogenous

Table 1. The CAM effector mechanism causing inhibition of mitochondrial respiration in L10 cells requires L-arginine or L-homoarginine and converts them to the carbamido-containing compounds L-citrulline and L-homocitrulline plus nitrite. (A) Carbamido compound concentration (micromoles per liter) after the first 24-hour incubation; (B) nitrite concentration (micromoles per liter) after the first 24-hour incubation; and (C) L10 cell death (percent) after the second 24-hour incubation in DMEM–G (*13*). To obtain activated macrophages, we infected C_3H/HeN female mice with BCG intraperitoneally as described (*9, 17*). Activated macrophages cultured in vitro in the presence of LPS are termed CAM. Stimulated macrophages were removed from uninfected normal mice inoculated with thioglycollate broth or peptone broth as described (*9, 17*). See (*13*) for description of the culture mediums used, cocultivation procedures, and documentation of inhibition of mitochondrial respiration. L10, a diethylnitrosamine-induced guinea pig (strain 2) hepatoma cell line was maintained in C-DMEM (*13*) plus 5% calf serum. L10 cells and peritoneal cells were tested periodically with the Mycotrin-TC mycoplasma test system (New England Nuclear) and were consistently negative for mycoplasma contamination. The L10 cells from each of the experimental groups cultured in DMEM–G were also cultured in DMEM+G (*13*) during the second 24-hour incubation. L10 cells cultured in DMEM+G maintained 98% or greater viability. L10 cells were also cocultivated with stimulated macrophages and remained >97% viable during a second incubation in DMEM–G. Carbamido compound and nitrite synthesis by stimulated macrophages + L10 cocultures was similar to that seen with stimulated macrophages cultured alone. Results are the mean ± SEM of three experiments.

First incubation additive concentration (m*M*)	Cytotoxic activated macrophages + L10 cells			L10 cells alone			Cytotoxic activated macrophages alone		Stimulated macrophages alone	
	A	B	C	A	B	C	A	B	A	B
	Carbamido compound	Nitrite	L10 death in DMEM–G	Carbamido compound	Nitrite	L10 death in DMEM–G	Carbamido compound	Nitrite	Carbamido compound	Nitrite
L-Arginine										
0	24.5 ± 1.2	22 ± 2.9	2 ± 0.4	13.4 ± 0.5	2 ± 1.3	2 ± 0.3	25.1 ± 0.6	22 ± 6.1	18.5 ± 1.2	3 ± 1.4
0.025	41.6 ± 5.7	40 ± 2.7	3 ± 0.5				36.5 ± 0.6	37 ± 3.5		
0.15	85.9 ± 3.6	59 ± 2.7	30 ± 2.5				92.5 ± 5.4	55 ± 5.5		
0.9	311.4 ± 11.5*	98 ± 6.6*	88 ± 1.9*	12.2 ± 0.5	2 ± 1.3	2 ± 0.3	276.4 ± 22.1*	92 ± 6.4*	21.7 ± 1.9	6 ± 2.5
L-Homoarginine										
0	20.2 ± 1.4	23 ± 2.2	2 ± 0.3	13.6 ± 1.3	3 ± 1.3	1 ± 0.3	20.0 ± 0.6	28 ± 3.3	15.9 ± 3.8	3 ± 1.4
0.025	37.2 ± 1.1	29 ± 2.7	2 ± 0.3				39.1 ± 1.7	34 ± 4.1		
0.15	145.9 ± 2.4	54 ± 2.7	25 ± 2.1				144.0 ± 1.4	59 ± 4.6		
0.9	323.3 ± 13.2*	66 ± 2.0*	74 ± 2.0*	14.7 ± 0.2	2 ± 1.3	2 ± 0.6	304.0 ± 18.1*	74 ± 4.1*	13.1 ± 0.3	4 ± 1.8
D-Arginine										
0	21.9 ± 4.3	23 ± 2.1	1 ± 0.3	13.4 ± 0.4	3 ± 1.3	1 ± 0.3	21.1 ± 3.6	27 ± 2.7	10.4 ± 0.4	3 ± 1.4
0.025	21.5 ± 3.4	20 ± 1.6	1 ± 0.4				20.2 ± 2.1	26 ± 2.8		
0.15	25.6 ± 2.3	19 ± 2.0	2 ± 0.3				17.5 ± 0.2	24 ± 2.3		
0.9	21.7 ± 0.6	21 ± 1.8	1 ± 0.5	14.1 ± 0.7	3 ± 1.3	1 ± 0.3	16.0 ± 0.8	24 ± 2.0	13.1 ± 0.5	3 ± 1.2

*$P < 0.001$ group cultivated or cocultivated in medium with 0.9 m*M* additive versus group cultivated or cocultivated with no additive (Student's *t* test).

Table 2. The CAM effector mechanism causing inhibition of aconitase and DNA synthesis in L10 cells requires L-arginine or L-homoarginine, converts L-arginine to L-citrulline plus nitrite, L-homoarginine to L-homocitrulline plus nitrite, and is inhibited by N^GMMA (N^G-monomethyl-L-arginine). CAM monolayers were prepared in Costar 3506 (35-mm) chambers as described (*9, 17*). L10 cells in the log phase of growth were added to CAM monolayers in DMEM–AA plus LPS (20 ng/ml), 5% dialyzed calf serum, and no further additives, or 1.2 m*M* L-arginine or 1.2 m*M* L-homoarginine, with or without 0.1 m*M* N^GMMA. The cocultivation was for the time indicated before assays were performed. Prior to ion exchange chromatography, serum proteins in the medium samples were precipitated with 10% trichloroacetic acid and removed by centrifugation (1000*g*). Results of the spectrophotometric aconitase assay are expressed as the rate of cis-aconitate disappearance in nanomoles per minute per 10^6 L10 cells. Aconitase activity and [^{3}H]thymidine uptake were measured as described (*7, 17*). Control L10 cells were cultured in medium with identical components as medium used for each group of L10 cells cocultivated with CAM and then assayed for aconitase or treated with [^{3}H]thymidine as described (*7, 17*). For other experimental details see (*13*) and the legend to Table 1. Values are the mean ± SEM of three experiments (citrulline, homocitrulline, and DNA synthesis; aconitase activity) and five experiments (nitrite synthesis). ND, not detected by the method.

Additives	Nitrite and citrulline or homocitrulline concentrations in coculture medium (μ*M*)						L10 cell aconitase activity and DNA synthesis			
	7.5 hours coculture		10 hours coculture		24 hours coculture		7.5 hours coculture		10 hours coculture	
							Aconitase activity		[^{3}H]thymidine uptake	
	Nitrite	Citrulline or homocitrulline	Nitrite	Citrulline or homocitrulline	Nitrite	Citrulline or homocitrulline	Nanomoles per minute per 10^6 cells	Percent of control L10 cells	Counts per minute	Percent of control L10 cells
A None	7.5 ± 1.6	ND	10.1 ± 0.82	ND	21.0 ± 1.68	ND	3.54 ± 0.48	81.5	16,097 ± 637	93.7
B L-Arginine	33.1 ± 0.36*	67.5 ± 7.5	44.8 ± 2.14*	111.4 ± 0.09	106.0 ± 3.00*	346.7 ± 46.5	0.30 ± 0.06†	7.6	897 ± 90*	6.0
C N^GMMA	3.1 ± 0.05	ND	3.1 ± 0.09	ND	4.3 ± 0.09	ND	4.06 ± 0.3	99.5	20,887 ± 739	138.2
D L-Arginine N^GMMA	11.4 ± 0.90	5.0 ± 3.0‖	15.8 ± 0.45	15.0 ± 9.0‡	24.2 ± 0.68	22.0 ± 18‖	3.16 ± 0.40	97.8	14,291 ± 1,310	99.4
E None	7.2 ± 0.55	ND	9.2 ± 0.77	ND	20.7 ± 1.68	ND	3.97 ± 0.53	101.5	15,220 ± 1,051	107.5
F L-Homoarginine	22.0 ± 0.95*	64.0 ± 6.1	32.9 ± 2.27*	103.3 ± 3.6	66.4 ± 5.82*	306.7 ± 60.9	0.23 ± 0.09*	5.3	2,680 ± 295*	20.6
G N^GMMA	3.3 ± 0.05	ND	3.2 ± 0.23	ND	3.7 ± 0.32	ND	4.10 ± 0.64	93.8	19,705 ± 1,390	158.2
H L-Homoarginine N^GMMA	7.6 ± 0.41	6.0 ± 2.0§	9.6 ± 0.64	14.2 ± 9.8§	21.6 ± 2.10	46.0 ± 6.0‖	3.53 ± 0.64	87.2	13,736 ± 850	116.6

*$P < 0.001$, †$P < 0.01$, nitrite synthesis, aconitase activity, or [^{3}H]thymidine uptake by group cocultivated with L-arginine or L-homoarginine versus group cocultivated with no additive (B versus A or F versus E). ‡$P < 0.001$, §$P < 0.01$, or ‖$P < 0.02$ L-citrulline synthesis by group cocultivated with L-arginine plus N^GMMA or L-homoarginine plus N^GMMA versus group cocultivated with N^GMMA alone (D versus B or H versus F).

substrate and serum was also significantly inhibited when compared to results with medium only ($P < 0.001$). Thus CAM-induced inhibition of aconitase activity and DNA synthesis, like inhibition of mitochondrial respiration, is dependent on an effector mechanism converting L-arginine to L-citrulline and nitrite. There was also a reproducible increase of [^{3}H]thymidine incorporation by L10 cells cultivated with CAM in culture medium containing N^GMMA but no L-arginine as compared to control L10 cells or L10 cultivated with CAM without additives (Table 2). Similar results were obtained with L-homoarginine, which was converted to L-homocitrulline with concurrent nitrite synthesis and similar biologic effects (Table 2).

Thin-layer chromatography (TLC) was used to detect L-[^{3}H]citrulline formed from L-[^{3}H]arginine by a CAM-associated deiminase (Table 3). CAM cells were cultured for 18 hours in medium with L-[^{3}H]arginine plus 0.4 m*M* unlabeled L-arginine, and lysates were prepared (Table 3). Sixty-six percent of the total ^{3}H in the lysates was associated with L-citrulline: (i) it had the same TLC migration pattern as the L-citrulline standard and (ii) the spot was scraped

Table 3. Detection by TLC and ion exchange chromatography of increased intracellular L-citrulline derived from L-arginine in CAM lysates. Macrophage monolayers were prepared in Costar 3506 chambers as described (*9, 17*). CAM cells were cultured in DMEM–AA + LPS (10 ng/ml) + 5% calf serum + L-[2,3-^{3}H]arginine (2 μ*Ci*/ml) and appropriate additives. L-[^{3}H]citrulline was measured in CAM cultured without target cells. See (*13*) for description of medium, culture, and coculture conditions. CAM monolayers were washed three times with phosphate-buffered saline after an 18-hour incubation, and TLC was carried out as described (*18*) with some modifications. Silica gel plates (10 × 20 cm, 200 μm thick) with a butanol-acetone-diethylamine-H_2O (36:36:7.2:18) solvent system were used. CAM cells were lysed with 0.2*N* NH_4OH, neutralized with CO_2, extracted with absolute ethanol, and evaporated to dryness; the residue was dissolved in 50 μl of water; and a 10-μl portion was applied to the TLC plate. After 90 minutes of development, each lane was divided into 5-mm sections, scraped into vials containing 200 μl of water plus 2 ml of Aquasol II, and quantitated in a scintillation counter. The location of the L-citrulline standard was determined by ninhydrin staining. Results are the mean ± SEM of ten experiments.

Additive	L-[^{3}H]Citrulline (%)
None	11.0 ± 0.78
0.4 m*M* L-arginine	66.4 ± 2.5*
0.05 m*M* N^GMMA	16.6 ± 2.0
0.4 m*M* L-arginine + 0.05 m*M* N^GMMA	25.7 ± 1.8

*$P < 0.001$, difference between CAM cultivated with 0.4 m*M* L-arginine alone and CAM cultivated with no additive.

from the TLC plate, dissolved in 0.1*N* sodium citrate buffer (*p*H 2.2), and confirmed to be L-citrulline by ion exchange chromatography. The intracellular accumulation of L-[^{3}H]citrulline was significantly less when the medium was not supplemented with unlabeled L-arginine or when 0.05 m*M* N^GMMA was added to the medium with 0.4 m*M* unlabeled L-arginine. Stimulated macrophages do not accumulate L-[^{3}H]citrulline intracellularly under the same experimental conditions. These findings demonstrate that, under conditions of culture that support the expression of cytotoxicity (for example, the presence of L-arginine and absence of N^GMMA), increased intracellular L-[^{3}H]citrulline derived from L-[^{3}H]arginine can be detected in CAM.

A pathway oxidizing imino nitrogen derived from L-arginine or L-homoarginine to nitrite (see Fig. 1) has not been described in either prokaryotes or eukaryotes. We propose that arginine deiminase is the first enzyme of this pathway and that it induces multiple metabolic changes in mammalian cells which, in addition to other effects, are capable of controlling cellular proliferation (*16*). It is also possible that this effector system participates in controlling the proliferation of some pathogenic microorganisms, particularly microbes capable of survival in the intracellular environment. The actual mechanism of metabolic inhibition by products of the L-arginine–dependent effector mechanism remains to be defined. However, nitrite or oxygenated nitrogen intermediates in the pathway of nitrite and nitrate synthesis could participate in causing iron loss from aconitase and other enzymes containing Fe-S clusters in target cells of CAM in addition to inducing other intracellular effects.

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The Cytoskeletal Protein Vinculin Contains Transformation-Sensitive, Covalently Bound Lipid

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Vinculin, which is associated with the cytoskeleton of many cells, has been suggested as a possible linker between microfilament bundles and the plasma membrane. Here it will be shown that fatty acid is covalently attached to vinculin in vivo. Furthermore, in chicken embryo fibroblasts infected with a temperature-sensitive mutant of Rous sarcoma virus, tsNY68, the acylation of vinculin at the permissive temperature was less than one-third that at the nonpermissive temperature. Thus, the covalent binding of lipid to vinculin is a transformation-sensitive event. The covalent modification of vinculin by lipids could be directly or indirectly involved in its reversible association with membranes. This modification may also provide a mechanism to alter the organization of vinculin within cells and thereby play a regulatory role in anchoring or stabilizing microfilament bundles at plasma membranes.

IN CULTURED FIBROBLASTS, MICROFILament bundles terminate at focal adhesion plaques, the specialized membrane regions where cells adhere most tightly to the underlying substratum (*1*). Vinculin is concentrated in these focal adhesion plaques (*2*) and thus may play a role in anchoring or stabilizing microfilament bundles at plasma membranes. Transformation of cells by oncogenic viruses leads to a reduction in the number of focal adhesion plaques and disrupts microfilament bundles concomitantly with markedly altering vinculin organization (*3, 4*). In cells infected with Rous sarcoma virus (RSV), a single gene product, $pp60^{src}$ (a protein kinase that modifies cellular proteins through phosphorylation on tyrosine residues), induces all these alterations (*5, 6*). Vinculin, one of the putative substrates of $pp60^{src}$, shows a marked transformation-dependent increase in phosphotyrosine content (*4*). However, it is believed that vinculin phosphorylation alone is not enough to explain the rearrangement of cytoskeletal elements observed during transformation (*6, 7*).

Recently it has been reported that α-actinin (*8, 9*), protein kinase C (*10*), and a few other proteins (*11*) are functionally regulated by noncovalent association with spe-

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