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Arginine Metabolism in Keratinocytes and Macrophages during Nitric Oxide Biosynthesis

MULTIPLE MODES OF ACTION OF NITRIC OXIDE SYNTHASE INHIBITORS

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ABSTRACT. Nitric oxide is an important cellular mediator produced in keratinocytes and macrophages from arginine by the enzyme nitric oxide synthase during inflammatory reactions in the skin. We found that γ-interferon stimulated nitric oxide production and the expression of inducible nitric oxide synthase in both cell types. However, macrophages produced more nitric oxide and nitric oxide synthase protein, and at earlier times than keratinocytes. Keratinocytes treated with γ-interferon took up more arginine than macrophages; however, they were less efficient in metabolizing this amino acid and exhibited reduced nitric oxide synthase enzyme activity. In both cell types, the nitric oxide synthase inhibitors, N^G-monomethyl-L-arginine (NMMA), L-N^5-(iminoethyl)ornithine, L-canavanine, and N^ω-nitro-L-arginine, as well as lysine, ornithine, and homoarginine markedly reduced arginine uptake. In contrast, N^ω-nitro-L-arginine methyl ester and N^ω-nitro-L-arginine benzyl ester were poor inhibitors of arginine uptake, while aminoguanidine had no effect on uptake of arginine by the cells. Moreover, NMMA was found to inhibit simultaneously arginine uptake and nitric oxide synthase enzyme activity in both cell types, whereas aminoguanidine only affected nitric oxide synthase activity. No major differences were observed between keratinocytes and macrophages. Taken together, these data demonstrate that, although keratinocytes and macrophages both synthesize nitric oxide, its production is regulated distinctly in these two cell types. Furthermore, in these cells, nitric oxide synthase inhibitors such as NMMA exhibit at least two sites of action: inhibition of nitric oxide synthase and cellular uptake of arginine. BIOCHEM PHARMACOL **54**;1:103–112, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. nitric oxide; keratinocytes; macrophages; aminoguanidine; γ-interferon; arginine; methylarginine

Nitric oxide is a potent vasoactive regulator, neuromodulator, and signaling molecule [1–3]. During inflammation, nitric oxide mediates cytotoxicity and nonspecific host defense [4]. In addition, nitric oxide regulates blood flow to the injured tissue. Excessive production of nitric oxide can lead to edema, prolonged inflammation, and injury by promoting the infiltration of macrophages and lymphocytes into the tissue [4]. Our laboratory has been interested in analyzing the role of nitric oxide in inflammatory reactions of the skin [5, 6]. During skin inflammation, infiltrating leukocytes are stimulated to produce γ-interferon [6, 7], a cytokine that modulates a variety of processes in epidermal cells including anti-viral activity, expression of major histocompatibility antigens, and cellular proliferation [5, 6]. Both keratinocytes and infiltrating macrophages synthesize nitric oxide in response to γ-interferon, and excessive production of this reactive mediator by these cells may promote tissue damage [3, 4]. Although the inducible (Type II) isoform of nitric oxide synthase has been purified, cloned, and characterized in macrophages [8], little information is available on the activity of the enzyme in keratinocytes. Keratinocyte-derived nitric oxide may play an important role in inflammation and in controlling microbial invasion and wound healing in the skin [5, 6].

Many studies have suggested that a reduction in nitric oxide synthase activity and subsequent nitric oxide production may be beneficial in abrogating inflammation [3, 4]. In this regard, inhibitors of nitric oxide have proven to be effective in reducing tissue damage in several inflammatory disease models [3, 4]. A question arises, however, about the extent to which these inhibitors are selective for nitric oxide synthase. Given the structural similarity of some of the inhibitors to arginine, it is possible that they also interfere with other cellular metabolic pathways that utilize this amino acid. Indeed, several nitric oxide synthase inhibitors have been reported to interfere with the cellular uptake of arginine as well as other neutral and basic amino acids [9–16]. In the present studies, we compared keratinocytes and macrophages with respect to their ability to

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produce nitric oxide. In addition, we examined the effects of various inhibitors on nitric oxide production and arginine uptake in these two cell types.

MATERIALS AND METHODS

Chemicals and Supplies

Tissue culture medium and all other cell culture supplies were obtained from GIBCO/BRL (Gaithersburg, MD). NIO† and NAME were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). L-[*ureido*-^{14}C]Citrulline (sp. act. 57 Ci/mmol) and L-[2,3-^{3}H]ornithine hydrochloride (sp. act. 53.4 Ci/mmol) were purchased from New England Nuclear (Wilmington, DE). L-[2,3,4,5-^{3}H]Arginine hydrochloride (sp. act. 60 Ci/mmol) was purchased from Amersham Life Sciences (Arlington Heights, IL). Amino acids, NMMA, NABE, arginine hydrochloride, and all other chemicals were of the highest purity available and were purchased from the Sigma Chemical Co. (St. Louis, MO). Recombinant murine γ-interferon was provided by Dr. Sidney Pestka, UMDNJ-Robert Wood Johnson Medical School (Piscataway, NJ). Antibody to inducible nitric oxide synthase was obtained from Affinity Bioreagents, Inc. (Golden, CO).

Cell Culture

PAM 212 cells are a keratinocyte cell line expressing both acidic and basic keratins [17] and were cultured as previously described [6]. RAW 264.7 macrophages were established from the ascites of tumor-bearing mice following intraperitoneal injection of Abelson leukemia virus [18, 19]. These cells were obtained from Dr. Donald Wolff, UMDNJ-Robert Wood Johnson Medical School. Cells were maintained in growth medium consisting of DMEM supplemented with 15% fetal bovine serum. For each experiment, keratinocytes or macrophages were inoculated into 24-well tissue culture plates (2.5×10^5 and 5×10^5 cells/well, respectively) in growth medium. After 24 hr, the medium was changed to phenol red- and serum-free DMEM with or without 100 U/mL of γ-interferon.

Assays for Nitric Oxide Production and Western Blotting of Nitric Oxide Synthase

Nitric oxide production by the cells was quantified spectrophotometrically by measuring the accumulation of nitrite in the culture medium using the Greiss reagent as previously described [6]. Briefly, at the indicated times, an aliquot of the culture medium was mixed with equal volumes of 1.0% sulfanilamide and 0.1% *N*-1-naphthylethylene diamine in 50% phosphoric acid. After 15 min at room temperature, the absorbance of the resulting chromophore was measured at 540 nm using a Perkin–Elmer Lambda 3 UV/VIS spectrophotometer and compared with standard solutions of sodium nitrite. Nitric oxide synthase expression in the cells was quantified by western blotting. Keratinocytes and macrophages were seeded into 6-well tissue culture plates (1×10^6 cells and 2×10^6 cells per well, respectively) and treated with 100 U/mL of γ-interferon for increasing time periods. The cells were then rinsed with HBSS (37°, pH 7.4), scraped off the plates into 1 mL of ice-cold 10 mM phosphate lysis buffer containing 0.1% SDS, 1 mM leupeptin, 1 mM pepstatin A, 0.1 mM aprotinin, 1 mM EDTA-Na_2 and 0.2 mM PMSF (pH 7.7), and sonicated on ice (3×10 sec, power setting 70, Artek Systems, Farmingdale, NY). Aliquots of cellular homogenates were mixed with an equal volume of Laemmli sample buffer, denatured at 95° for 10 min in an oil bath, and then centrifuged for 10 min at 10,000*g*. For each cell type, equal amounts of total cellular protein was loaded onto 7.5% SDS–polyacrylamide gels, electrophoresed using the Laemmli buffer system, and then transferred to a nitrocellulose membrane. Nonspecific antibody binding was blocked by incubating the membrane for 1 hr with 5% nonfat dried milk protein in 20 mM Tris-HCl-buffered saline (pH 7.4) containing 0.1% Tween 20. Then the membrane was incubated for 2 hr with a 1:2000 dilution of the inducible nitric oxide synthase-specific antibody (Upstate Biotechnology Inc., Lake Saranac, NY) followed by a 1-hr incubation with a 1:10,000 dilution of horseradish-peroxidase-conjugated anti-rabbit secondary antibody. Inducible nitric oxide synthase was visualized on the blots using a chemiluminescence-based detection kit and X-ray film (ECL Western Blotting Kit, Amersham Life Sciences). Protein content in cellular homogenates was determined by the method of Bradford [20] using bovine serum albumin as the standard.

[^{3}H]Arginine Uptake Studies

γ-Interferon-treated keratinocytes or macrophages in 24-well tissue culture plates were washed rapidly with uptake buffer (5×1 mL, 37°) consisting of HBSS supplemented with 20 mM HEPES. Uptake buffer (200 μL) containing a 10 μM concentration of carrier-labeled [^{3}H]arginine (500,000 cpm per well) with and without various inhibitors was then added to the wells. After appropriate incubation periods at 37°, uptake was terminated by aspirating the labeling medium and rapidly washing the cells with ice-cold uptake buffer (5×1 mL). To measure total [^{3}H]-arginine uptake, the cells were solubilized by the addition of 0.5 mL of 0.2 N NaOH to each well. After 30 min, aliquots of the solubilized cells were transferred to plastic scintillation vials and counted for radioactivity. To measure uptake of [^{3}H]-arginine into acid-soluble and acid-insoluble cellular fractions, cells were incubated with [^{3}H]arginine as described above. Following the uptake period and washes, the tissue

† *Abbreviations:* AG, aminoguanidine; AMAIB, α-methylamino)isobutyric acid; CAN, L-canavanine; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hanks' balanced salt solution; NA, N^{ω}-nitro-L-arginine; NABE, N^{ω}-nitro-L-arginine benzyl ester; NAME, N^{ω}-nitro-L-arginine methyl ester; NIO, L-N^{5}-(iminoethyl)ornithine; NMMA, N^{G}-monomethyl-L-arginine; and TCA, trichloroacetic acid.

culture plates were placed on ice and 0.5 mL of ice-cold 5% TCA was added to each well. After 20 min, the TCA-soluble material containing the free cytosolic amino acids was removed, and an aliquot was counted for radioactivity or analyzed by HPLC as described below. The remaining acid-insoluble precipitated material in each well was washed gently with 5% ice-cold TCA (3 × 1 mL) and then solubilized by the addition of 0.5 mL of 0.2 N NaOH. After an additional 30 min, an aliquot of the NaOH-soluble material was removed and counted for radioactivity. Under these conditions no cellular protein was detected in the TCA extracts containing the free cytosolic amino acids, and greater than 98% of the total cellular protein was recovered in the NaOH-solubilization step. In addition, > 90% of [^{3}H]arginine uptake into acid-insoluble material was blocked by 10 μg/mL of cycloheximide, demonstrating that uptake was largely due to incorporation of [^{3}H]arginine into cellular proteins.

HPLC Analysis of [^{3}H]Arginine Metabolism and Nitric Oxide Synthase

[^{3}H]Arginine and its metabolites were analyzed in the TCA extracts of macrophages and keratinocytes using HPLC with radiometric detection. Aliquots of the TCA-soluble extracts were centrifuged at 15,000*g* (4°, 10 min) and then injected into a Waters gradient HPLC system connected to a Whatman EQC ion exchange column (250 × 4.6 mm). The samples were eluted at a flow rate of 1 mL/min by a step gradient of mobile phase A (185 mM citric acid monohydrate, 15 mM sodium citrate, pH 2.3) and mobile phase B (200 mM sodium citrate, pH 8.8) as follows: 100% A for 0 to 6.0 min, 50% A and 50% B from 6.1 to 15 min, a linear gradient from 50% A and 50% B to 100% B from 15.1 to 21 min, followed by 100% B from 21.1 to 25 min. After elution of the metabolites, the column was re-equilibrated with 100% A for 10 min. Peaks were detected with a Packard Radiomatic model A110 on-line scintillation counter equipped with a 500-μL flow cell using a flow rate of 3 mL/min for the scintillation fluid (Monoflow 5, National Diagnostics, Atlanta, GA). Under these conditions, the counting efficiency for [^{3}H]arginine and [^{3}H]citrulline was 32%. The retention times for citrulline, ornithine, and arginine were 6.1, 18.5, and 21.1 min, respectively.

RESULTS

Comparison of Nitric Oxide Synthase Expression in Keratinocytes and Macrophages

Initially, keratinocytes and macrophages were compared with respect to nitric oxide synthase expression and nitric oxide production. Figure 1 (top panel) demonstrates that γ-interferon (100 U/mL) stimulated expression of the inducible isoform of nitric oxide synthase in both cell types. Expression of this protein in macrophages was evident 12 hr after treatment with γ-interferon, peaked at 24 hr, and declined thereafter. In contrast, expression of the enzyme in keratinocytes increased continuously over the 48-hr incubation period. At 24 hr, greater amounts of the enzyme protein were present in macrophages when compared with keratinocytes. Nitric oxide production, measured as the accumulation of nitrite in the cell culture medium, was evident in both cell types within 12–24 hr and increased continuously over 48 hr (Fig. 1, bottom panel). Greater amounts of nitrite were produced by macrophages than by keratinocytes.

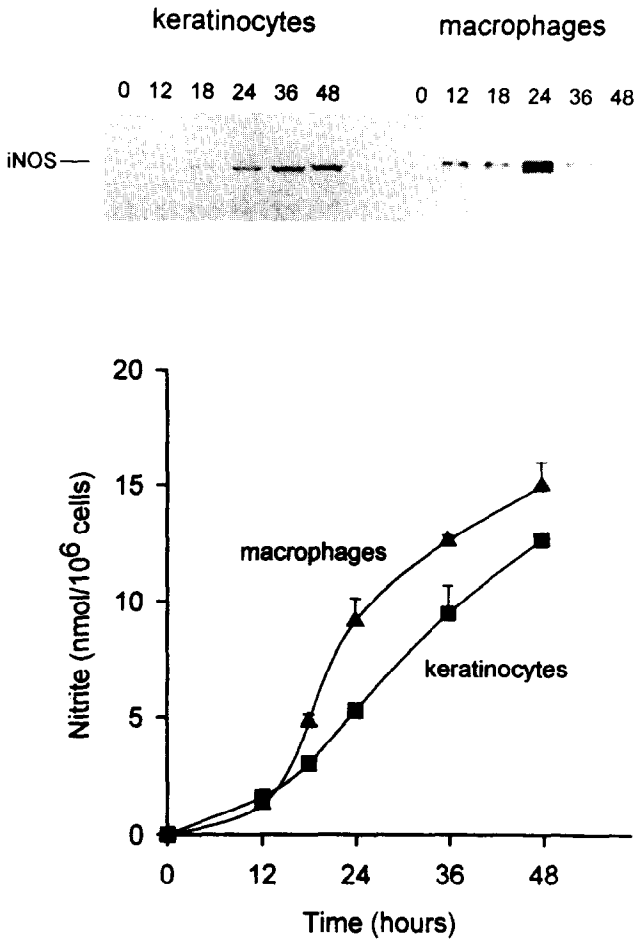

FIG. 1. Expression of nitric oxide synthase and nitric oxide production by keratinocytes and macrophages. Top panel: expression of nitric oxide synthase protein in keratinocytes and macrophages. Extracts of cells were prepared after treatment with γ-interferon (100 U/mL) and analyzed by western blotting using an antibody to inducible nitric oxide synthase. Bottom panel: nitrite production by keratinocytes and macrophages treated with γ-interferon. Nitric oxide production was measured as the accumulation of nitrite in the culture medium as described in Materials and Methods.

Uptake and Metabolism of [^{3}H]Arginine

[^{3}H]Arginine was found to be taken up rapidly by both keratinocytes and macrophages and accumulated in the cytoplasm (acid-soluble material) and protein (acid-insol-

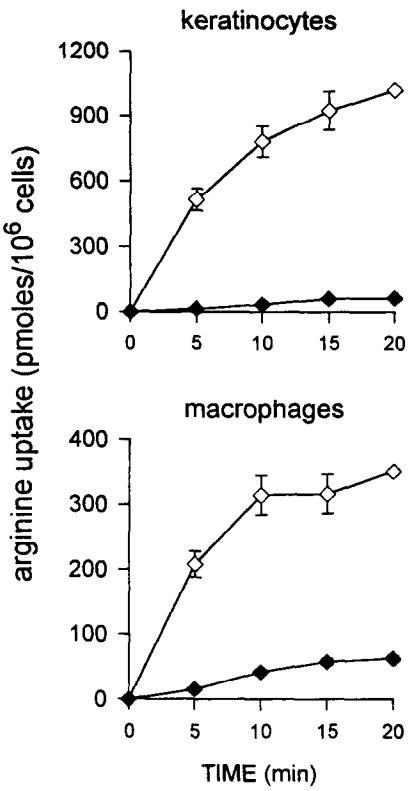

FIG. 2. Uptake of arginine into keratinocytes and macrophages. Keratinocytes and macrophages were pulse-labeled with [^{3}H]arginine for increasing periods of time. The distribution of ^{3}H-labeled amino acids in acid-soluble and -insoluble fractions of the cells was then determined. Open symbols: accumulation of ^{3}H-material into acid-soluble cellular fractions; closed symbols: accumulation of ^{3}H-material into acid-insoluble cellular fractions. Each point represents the mean ± SD of triplicate samples.

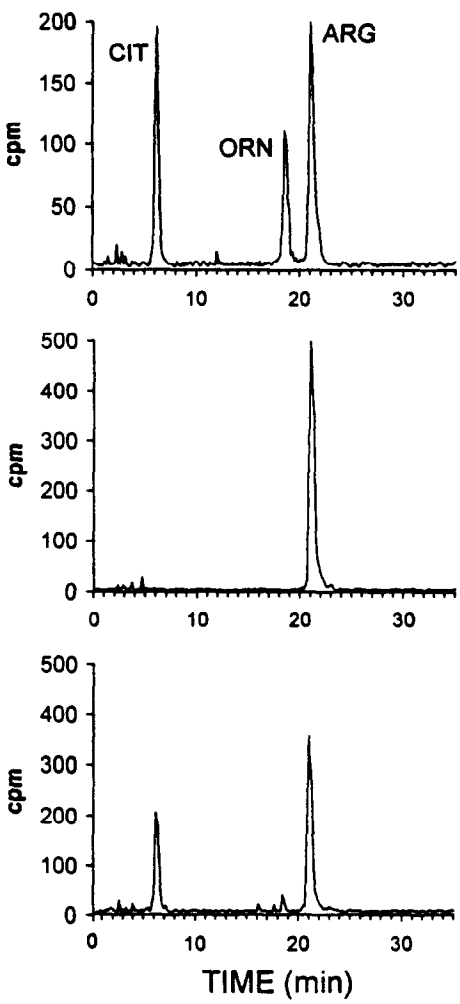

FIG. 3. Amino acid analysis of acid soluble pools of keratinocytes. Acid-soluble extracts of keratinocytes incubated with [^{3}H]arginine were analyzed by HPLC with radiometric detection. Top panel: separation of citrulline (CIT), ornithine (ORN), and arginine (ARG) standards. Center panel: analysis of control keratinocytes pulse-labeled with [^{3}H]arginine for 10 min. Bottom panel: analysis of keratinocytes treated with γ-interferon (100 U/mL) for 24 hr and then pulse-labeled with [^{3}H]arginine for 10 min. Note that [^{3}H]arginine was metabolized almost exclusively to [^{3}H]citrulline in γ-interferon-treated cells.

uble material) of the cells (Fig. 2). Under our assay conditions, approximately 5–15% of the label was found to incorporate into protein in each cell type. Total accumulation of [^{3}H]arginine in keratinocytes was greater than in macrophages. Amino acid analysis of the acid-soluble fractions of the cells revealed that metabolism of [^{3}H]arginine in control cells was minimal (Figs. 3, 4A, and 4B). Thus, 90–95% of the label remained as [^{3}H]arginine in the acid-soluble pools of the cells, while only 5–10% was metabolized to [^{3}H]citrulline and [^{3}H]ornithine. In contrast, in γ-interferon-treated cells, metabolism of [^{3}H]arginine to [^{3}H]citrulline was increased markedly, while no major changes in the formation of [^{3}H]ornithine were observed (Fig. 4, C and D). Rapid metabolism of arginine to citrulline under our experimental conditions indicates a high turnover rate for cytosolic pools of arginine utilized for nitric oxide biosynthesis. The fact that only small amounts of ornithine were detected suggests that arginase activity in these cells is relatively low.

The metabolism of [^{3}H]arginine was greater in macrophages than in keratinocytes. Consequently, the rate of formation of citrulline in macrophages was greater (Fig. 5).

The relatively high rate of [^{3}H]arginine metabolism in macrophages 24 hr after treatment with γ-interferon is likely due to the large amounts of nitric oxide synthase protein in the cells at this time (Fig. 1, top panel).

Effects of Inhibitors on Uptake and Metabolism of [^{3}H]Arginine

Several amino acids as well as arginine analogs known to inhibit nitric oxide synthase, including AG, NABE, NAME, NA, NIO, CAN, and NMMA, were next compared for their effects on [^{3}H]arginine uptake into the cells under standard conditions (Fig. 6). In keratinocytes, arginine uptake was not affected by citrulline, AG, or AMAIB,

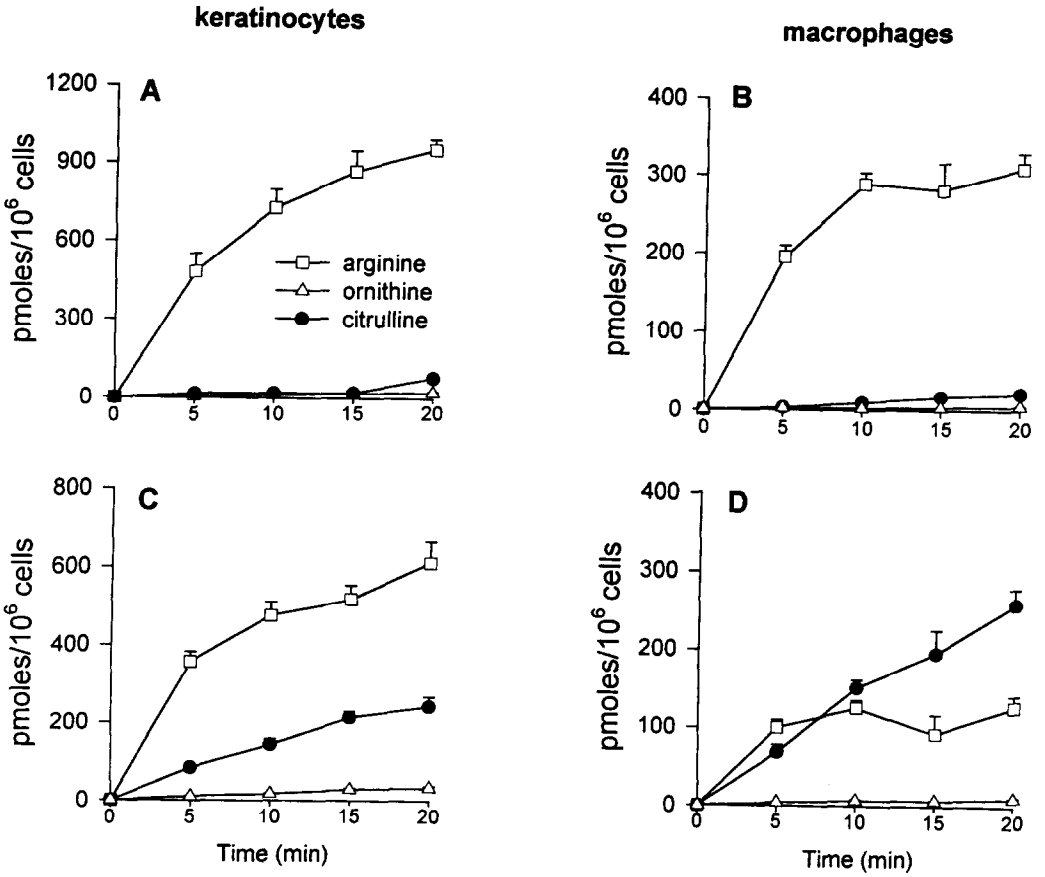

FIG. 4. Metabolism of [^{3}H]arginine in keratinocytes and macrophages. Levels of [^{3}H]arginine, [^{3}H]ornithine, and [^{3}H]citrulline in acid-soluble pools of keratinocytes and macrophages pulse-labeled with 10 μM [^{3}H]arginine were quantified using HPLC with radiometric detection. Panels A and B: metabolism of [^{3}H]arginine in control keratinocytes and macrophages, respectively; panels C and D: metabolism of [^{3}H]arginine in keratinocytes and macrophages treated with γ-interferon for 24 hr. Each point represents the mean ± SEM of triplicate samples.

a selective inhibitor of the A amino acid transporter [21–23]. Alanine, serine, and cysteine, the prototypical substrates for the ASC transport system [22, 23], only weakly inhibited [^{3}H]arginine uptake. Although NABE and NAME were also poor uptake inhibitors, NA, leucine, and isoleucine reduced arginine uptake by 40–60% of control. This was surprising since all five of these compounds have been characterized previously as substrates for the L transport system [22, 23]. Thus, there appears to be heterogeneity in the transport systems used by these compounds. The cationic nitric oxide synthase inhibitors NMMA, NIO, and CAN reduced [^{3}H]arginine uptake by 70–80%, while lysine and ornithine inhibited uptake by 50–60% (Fig. 6). Homoarginine (HA) was the most potent inhibitor, reducing [^{3}H]arginine uptake by approximately 85%. Figure 6 also shows the concentration dependence of some classic nitric oxide synthase inhibitors on [^{3}H]arginine uptake in keratinocytes. NMMA ($IC_{50} \approx 90$ μM) was the most potent inhibitor followed by NIO ($IC_{50} \approx 300$ μM) and NA ($IC_{50} \approx 900$ μM). AG, NAME, and NABE had little or no effect on [^{3}H]arginine uptake at concentrations up to 3 mM (Fig. 6 and data not shown). Taken together, these data indicate that arginine uptake in keratinocytes occurs primarily through a y^+ transport system [22, 23]. In general, similar patterns of inhibition of [^{3}H]arginine uptake were observed in macrophages (not shown). In addition, the patterns of inhibition in untreated and γ-interferon-treated cells were nearly identical. However, the total uptake of [^{3}H]arginine was 30–50% greater in both keratinocytes and macrophages following a 24-hr treatment with γ-interferon (data not shown). Enhanced uptake of [^{3}H]arginine in macrophages following γ-interferon treatment has been described previously [24–26]. Based on our data, it appears that several of the nitric oxide synthase inhibitors examined interfere with uptake of arginine into both keratinocytes and macrophages.

Since nitric oxide synthase inhibitors can block the uptake of [^{3}H]arginine into cells, a question arises as to whether these compounds act by limiting supplies of intracellular arginine or by inhibiting nitric oxide synthase. To address this question, we compared arginine uptake (NMMA and NA) and non-uptake (AG) inhibitors for their ability to suppress nitric oxide synthase activity in intact cells stimulated with γ-interferon. To measure nitric oxide synthase enzyme activity without interference by uptake inhibition, cells were pretreated with the inhibitors,

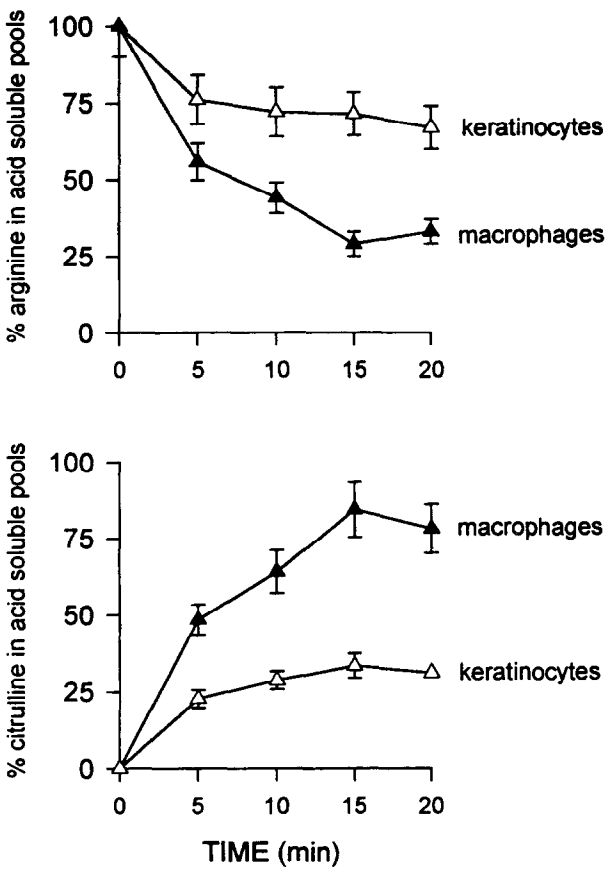

FIG. 5. Distribution of [^{3}H]arginine and [^{3}H]citrulline in keratinocytes and macrophages. Top panel: time-dependent alterations in the percentage of [^{3}H]arginine in acid-soluble pools of keratinocytes and macrophages. Bottom panel: time-dependent alterations in the percentage of [^{3}H]citrulline in acid-soluble pools of keratinocytes and macrophages. See Fig. 4 for control values. Values are means ± SD, N = 3.

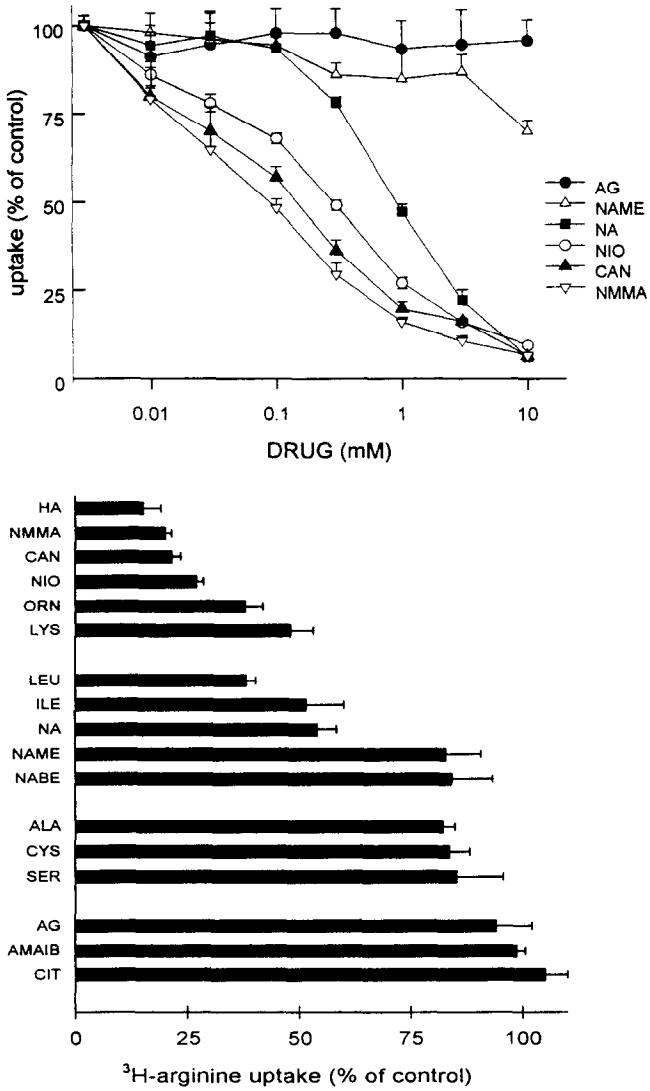

FIG. 6. Comparison of the effects of various enzyme inhibitors on arginine uptake into keratinocytes. Top panel: cells coincubated for 10 min with 10 μM [^{3}H]arginine in the presence of increasing concentrations of the nitric oxide synthase inhibitors. Bottom panel: cells coincubated for 10 min with 10 μM [^{3}H]arginine and various amino acids and arginine analogs (1 mM). HA = homoarginine. Each point represents the mean ± SD of triplicate samples. Control uptake was 243 ± 19 pmol/10^6 cells.

washed, and then incubated with [^{3}H]arginine. Table 1 shows that nitric oxide synthase activity, as measured by the ability of the cells to metabolize [^{3}H]arginine to [^{3}H]citrulline, was suppressed in both keratinocytes and macrophages by preincubation with the inhibitors. NMMA, NA, and AG were less effective inhibitors of keratinocyte nitric oxide synthase activity than of macrophage enzyme activity (Table 1). Table 2 shows that, under preincubation conditions, neither NMMA nor AG altered arginine uptake by the cells, but each markedly inhibited nitric oxide synthase activity and [^{3}H]citrulline production. When coincubated with [^{3}H]arginine, both AG and NMMA inhibited nitric oxide synthase, as evidenced by the decrease in the extent of metabolism of [^{3}H]arginine to [^{3}H]citrulline (Table 3). However, NMMA, but not AG, also inhibited arginine uptake. The concentration dependence of the inhibitory effects of NMMA on nitric oxide synthase activity and arginine uptake is shown in Fig. 7. From this figure it is apparent that both of these processes occur simultaneously, and decreased [^{3}H]citrulline produced in cells coincubated with NMMA thus represents both inhibition of [^{3}H]arginine uptake and nitric oxide synthase inhibition. Under these coincubation conditions, reduced arginine uptake largely accounted for the decrease in citrulline production by the cells. In contrast to NMMA, AG did not inhibit [^{3}H]arginine uptake at concentrations up to 3 mM, and decreased citrulline production in the presence of this drug was due primarily to inhibition of nitric oxide synthase activity (Fig. 7).

The arginine uptake inhibitors lysine and ornithine, which do not inhibit the activity of nitric oxide synthase, were also examined. When cells were preincubated with these amino acids, [^{3}H]arginine uptake was increased (Table 2). Although these amino acids did not directly affect

TABLE 1. Effects of nitric oxide synthase inhibitors on the production of citrulline by keratinocytes and macrophages*

	[^{3}H]Citrulline (pmol/10^6 cells)	
Inhibitors†	Keratinocytes	Macrophages
None	242.9‡	260.2
NMMA	30.8 (87.3)§	13.0 (95.0)
NA	28.4 (88.3)	15.1 (94.2)
AG	71.6 (70.5)	23.4 (91.0)

* Cells were treated for 24 hr with 100 U/mL of γ-interferon prior to assays for nitric oxide synthase activity.

† Cells were pretreated with buffer control or the inhibitors (2 mM). After 20 min, the cells were rinsed, pulsed-labeled with 10 μM [^{3}H]arginine for 10 min, and then assayed for [^{3}H]citrulline production by HPLC with radiometric detection as described in Materials and Methods.

‡ Mean of triplicate samples, SD ≤ 10%.

§ Percentage inhibition of [^{3}H]citrulline production.

nitric oxide synthase activity in the cells, there was a small increase in [^{3}H]citrulline production (Table 2). This can be attributed entirely to increased arginine uptake by the cells. When coincubated with [^{3}H]arginine, lysine and ornithine inhibited [^{3}H]citrulline production by the cells despite the fact that they did not affect nitric oxide synthase activity (Table 3). These effects were concentration dependent up to 3 mM (Fig. 7). Thus, arginine uptake inhibitors by themselves can be effective inhibitors of nitric oxide production.

DISCUSSION

Among the many factors that determine if keratinocytes or macrophages produce nitric oxide is the amount of enzymatically active nitric oxide synthase in the cells and the availability of enzyme substrates and cofactors [1–4]. Thus, although γ-interferon stimulated expression of inducible nitric oxide synthase and nitric oxide production in both cell types, macrophages expressed nitric oxide synthase at earlier times than keratinocytes. In addition, the kinetics of enzyme protein production were distinct. In keratinocytes, nitric oxide synthase expression increased continuously over time, whereas its expression in macrophages was transient. These differences may be due to greater expression of translatable mRNA for inducible nitric oxide synthase in macrophages, which could explain why these cells contain more enzyme protein [7]. Transient expression of nitric oxide synthase in macrophages may be due to changes in the rate of synthesis and/or degradation of enzyme protein or mRNA over time in culture. The fact that macrophages produced more nitric oxide than keratinocytes and were more efficient in converting arginine to citrulline (see further below) indicates that the cells contain more nitric oxide synthase enzyme activity.

Arginine was found to be rapidly taken up and metabolized by both keratinocytes and macrophages. In pulse-labeling experiments, keratinocytes were found to accumulate more arginine than macrophages did. In most mammalian cells, arginine uptake occurs by the well characterized y^+ cationic amino acid transporter [21–23]. Arginine transport in many cell types is up-regulated following treatment with γ-interferon or endotoxin [24–26] which may be required to increase available arginine for nitric oxide biosynthesis during infection and inflammation. Differences between keratinocytes and macrophages with respect to arginine uptake may be due to unique aspects of regulation, expression, and/or kinetic characteristics of the y^+ transport protein [27]. In this regard, recent evidence indicates that the kinetic characteristics of basic amino acid uptake in macrophages and endothelial cells are distinct [9, 11, 12, 14, 15]. In our studies, clear differences in arginine metabolism were noted between control and γ-interferon-treated cultures of keratinocytes and macrophages, and this was largely due to the appearance of nitric oxide synthase activity in the treated cells. This conclusion is based upon our findings that the major metabolite of arginine in both cell types after γ-interferon treatment was citrulline and the fact that citrulline and nitric oxide production were readily inhibited by nitric oxide synthase inhibitors. Macrophages were found to be more efficient than keratinocytes in metabolizing [^{3}H]arginine to [^{3}H]citrulline, which is likely due to greater nitric oxide synthase enzyme activity.

TABLE 2. Effects of preincubation of keratinocytes with inhibitors on uptake and metabolism of [^{3}H]arginine*

Inhibitors†	[^{3}H]Arginine uptake (% of control)	Nitric oxide synthase activity (% conversion to [^{3}H]citrulline)	[^{3}H]Citrulline production (pmol/10^6 cells)
None	100.0‡	56.6	196
AG	99.4	8.1	28
NMMA	94.5	7.8	25
LYS	145.1	48.1	241
ORN	130.5	52.0	235

* Cells were treated for 24 hr with 100 U/mL of γ-interferon prior to assays. Arginine uptake is presented as a percentage of control uptake in the absence of inhibitors. Nitric oxide synthase activity is presented as the percentage of [^{3}H]arginine in the cells converted to [^{3}H]citrulline during the pulse-labeling period. Control [^{3}H]arginine uptake was 346 pmol/10^6 cells. In the absence of inhibitors, control [^{3}H]citrulline formed (nitric oxide synthase activity) was 196 pmol/10^6 cells.

† Cells were pretreated with buffer control or the inhibitors (1 mM). After 30 min, the cells were rinsed, pulsed-labeled with 10 μM [^{3}H]arginine for 10 min, and then assayed for arginine uptake and nitric oxide synthase activity as described in Materials and Methods.

‡ Mean of triplicate samples, SD ≤ 10%.

TABLE 3. Effects of coincubation of inhibitors on [^{3}H]arginine uptake and metabolism by keratinocytes*

Inhibitors†	[^{3}H]Arginine uptake (% of control)	Nitric oxide synthase activity (% conversion to [^{3}H]citrulline)	[^{3}H]Citrulline production (pmol/10^6 cells)
None	100.0‡	68.1	211
AG	104.5	13.1	42
NMMA	32.2	18.4	18
LYS	48.9	67.5	102
ORN	37.7	62.0	72

* Cells were treated for 24 hr with 100 U/mL of γ-interferon prior to assays. Arginine uptake is presented as a percentage of control uptake in the absence of inhibitors. Nitric oxide synthase activity is presented as the percentage of [^{3}H]arginine in the cells converted to [^{3}H]citrulline during the pulse-labeling period. Control [^{3}H]arginine uptake was 310 pmol/10^6 cells. In the absence of inhibitors, control [^{3}H]citrulline formed (nitric oxide synthase activity) was 211 pmol/10^6 cells.

† Cells were treated with 10 μM [^{3}H]arginine for 10 min in the presence and absence of inhibitors (1 mM) and then assayed for arginine uptake and nitric oxide synthase activity as described in Materials and Methods.

‡ Mean of triplicate samples, SD ≤ 10%.

In these experiments, cells were pulsed with 10 μM [^{3}H]arginine; however, similar results were found using concentrations of [^{3}H]arginine up to 50 μM (unpublished studies). Under typical cell culture conditions, the medium in which the cells are maintained is supplemented with up to 1.6 mM arginine. This results in high intracellular arginine levels that can alter the activity of other enzymes involved in arginine metabolism [28]. The extracellular concentrations of arginine used in our experiments more closely reflect levels of arginine in physiological fluids.

Almost all mammalian cells obtain arginine from extracellular sources. Thus, levels of intracellular arginine and nitric oxide production will be highly sensitive to the supply of this amino acid [28], a process that can be modified by arginine uptake inhibitors. The present studies demonstrated that, in coincubation experiments, several inhibitors of nitric oxide synthase, in particular NMMA and NIO, as well as the cationic amino acid homoarginine, effectively block uptake of extracellular arginine into keratinocytes and macrophages. These findings are consistent with earlier reports that these compounds directly compete with arginine for the y^+ transporter [9, 10, 12–15]. Inhibition of arginine uptake was not observed when cells were preincubated with the inhibitors prior to measuring arginine uptake. Some overlap in substrate specificities for amino acid transport can account for our findings that leucine and isoleucine, as well as NA, inhibited arginine uptake at high inhibitor:arginine ratios (100:1) (see Refs. 21–23 for reviews). These amino acids, as well as NABE, NAME, and citrulline, have been classified as substrates for the L transport system, which is responsible for uptake of aliphatic branched chain amino acids [14–16, 21–23]. In contrast, alanine, serine, and cysteine, which define the ASC amino acid transport system, were found to be poor inhibitors of arginine uptake into keratinocytes and macrophages.

Decreased nitric oxide production in keratinocytes treated with NMMA was found to be due to a combination of reduced arginine uptake and inhibition of nitric oxide synthase. However, when low concentrations of arginine were used, inhibition of uptake of this amino acid was largely responsible for decreased citrulline production. The contribution of reduced arginine uptake to impaired nitric oxide production depends on the relative extracellular concentrations of the inhibitor and arginine, as well as competition by other basic amino acids at the y^+ transporter (see further below). Nitric oxide synthase inhibitors such as NMMA also enter cells via the y^+ transporter, and high extracellular concentrations of cationic amino acids may interfere with their uptake into target cells [14]. In our studies comparing enzymatic inhibitors of nitric oxide synthase, NMMA was the most potent inhibitor of arginine uptake possibly due to high affinity of this arginine analog for the y^+ transporter.

The fact that compounds such as NMMA inhibit cellular arginine uptake may be an undesirable effect *in vivo* since this amino acid is required for protein synthesis and is a precursor for numerous physiologically active molecules including polyamines [29, 30]. Reduced availability of amino acids can lead to growth inhibition and cytotoxicity [29, 31, 32]. This could be important at sites of inflammation and injury where localized nitric oxide synthase, and arginase released by tissue macrophages, can deplete extracellular arginine [33–35]. Our findings that AG effectively inhibits nitric oxide synthase in cells without altering arginine uptake may thus be particularly relevant and could lead to better uses of this drug which is apparently more selective in antagonizing nitric oxide mediated pathology. AG is known to be selective for the inducible form of nitric oxide synthase [36, 37] and has been explored as a therapeutic agent in disease models thought to be mediated by nitric oxide such as immunologically induced diabetes [38]. In addition, unlike other nitric oxide synthase inhibitors, AG also does not interfere with substrates for the L amino acid transporter and its uptake into cells is not saturable (unpublished studies). However, AG does inhibit amine oxidases as well as non-enzymatic glycosylation [39, 40], and the role of these reactions in regulating nitric oxide synthase activity is not known at the present time. Of interest were our findings that coincubation of cells with

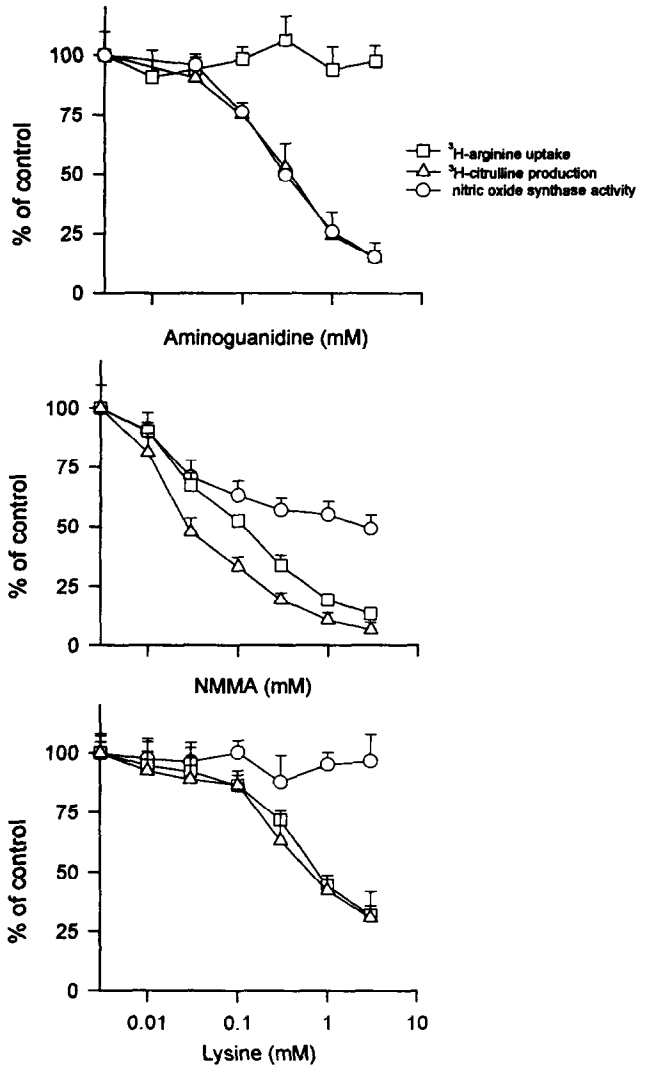

FIG. 7. Effects of inhibitors of nitric oxide on [^{3}H]arginine uptake, [^{3}H]citrulline production, and nitric oxide synthase activity in murine keratinocytes. γ-Interferon-treated cells were coincubated with increasing concentrations of the nitric oxide synthase inhibitors and [^{3}H]arginine (10 μM) for 10 min and then analyzed for uptake of [^{3}H]arginine, nitric oxide synthase activity, and [^{3}H]citrulline production. Top panel: cells treated with AG; center panel: cells treated with NMMA; bottom panel: cells treated with lysine. Each point represents the mean ± SD of triplicate samples. In γ-interferon-treated cells, control [^{3}H]arginine uptake was 346 pmol/10^6 cells, control [^{3}H]citrulline production was 196 nmol/10^6 cells, and control nitric oxide synthase activity was 19.6 pmol/min/10^6 cells.

lysine or ornithine, in the presence of arginine, inhibited citrulline production by the cells. Although lysine and arginine do not inhibit nitric oxide synthase, they do reduce arginine accumulation in cells by competing for the y^+ transporter [21, 22], and this process alone can result in decreased nitric oxide production [11]. When cells were pretreated with lysine or ornithine, arginine uptake increased, resulting in enhanced citrulline production. Increased uptake of arginine is likely due to *trans*-stimulation of the y^+ transporter by lysine or ornithine efflux when the cells were rinsed free of these amino acids and then pulsed with [^{3}H]arginine [21].

In conclusion, the present studies demonstrated that, in response to γ-interferon, keratinocytes and macrophages are distinct with respect to their ability to produce nitric oxide, express nitric oxide synthase protein and enzyme activity, and accumulate arginine. In addition, in these cell types, there are different classes of inhibitors of nitric oxide production, some of which function not only by inhibiting nitric oxide synthase, but also by reducing available supplies of arginine. At the present time, it is not clear which of these sites is responsible for reducing nitric oxide production *in vivo*. Further studies are required to understand the mechanism of action of nitric oxide synthase inhibitors in skin cells since regulation of nitric oxide production by keratinocytes and macrophages may be important in limiting inflammation and promoting wound healing [5, 6]. It should be noted that the keratinocytes and macrophages used in the present studies are defined cell lines. Previous work has shown that primary cultures of both keratinocytes and macrophages produce nitric oxide [6–8], and further studies are needed to determine if the cell lines and primary cultures respond in a similar manner to the nitric oxide synthase inhibitors.

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