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Apolipoprotein E acts to increase nitric oxide production in macrophages by stimulating arginine transport

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Abstract

Previous studies have shown that apolipoprotein E (apoE) plays a role in immune function by modulating tissue redox balance. Using a mouse macrophage cell line (RAW 264.7), we have examined the mechanism by which apoE regulates nitric oxide (NO) production in macrophages. ApoE potentiates NO production in immune activated RAW cells in combination with lipopolysaccharide or polyinosinic:polycytidylic acid (PIC), agents known to induce expression of inducible nitric oxide synthase mRNA and protein. The effect is not observed with apolipoprotein B or heat-inactivated apoE. The combination of PIC plus apoE produced more NO than the level expected from an additive effect of PIC and apoE alone. Furthermore, this increase was observed at submaximal extracellular arginine concentrations, suggesting that apoE altered arginine (substrate) availability. Examination of [^{3}H]arginine uptake across the cell membrane demonstrated that arginine uptake was increased by PIC but further increased by PIC plus apoE. Treatment of RAW cells with apoE was associated with an increased apparent V_{max} and decreased affinity for arginine as well as a switch in the induction of mRNA for subtypes of cationic amino acid transporters (CAT). Treatment of RAW cells with PIC plus apoE resulted in the loss of detectable CAT1 mRNA and expression of CAT2 mRNA. Regulation of arginine availability is a novel action of apoE on the regulation of macrophage function and the immune response.

Keywords: Arginine transport; Apolipoprotein E; Macrophage; Nitric oxide; Lipid; Alzheimer's disease

1. Introduction

Apolipoprotein E (apoE) is the protein component of a lipid-protein complex and is a member of a larger family of apoproteins that transports lipids throughout the body. Although apoE is found in plasma, it is a primary component of lipid and cholesterol metabolism in the central nervous system (CNS) and is particularly important during synthesis and repair of glial and neuronal membranes. ApoE expression is increased during the response of the CNS to injury and has been observed to promote neuron sprouting [1–5]. The function of apoE in neuronal injury and repair, however, extends beyond regulation of lipid transport and the supply of lipids for membrane reconstruction. Previous studies have demonstrated that apolipoproteins can alter the tissue redox balance. Miyata and Smith [6] have shown

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that apoE serves as a direct antioxidant and can protect cells from oxy radical-mediated damage. Furthermore, mice lacking apoE (APOE−/−) demonstrated decreased antioxidant levels and decreased recovery after head injury [7]. We have shown that apoE increased the production of nitric oxide (NO) from human monocyte-derived macrophages (MDM) [8]. Macrophages are a major cellular source of NO and are stimulated to produce NO and other reactive oxygen species by proinflammatory factors [9–11]. These reactive species are part of the innate immune response mediated by macrophages during tissue inflammation and injury [11–14]. Thus, apoE-mediated regulation of NO may be an important link between tissue redox balance and inflammation. The link between NO and apoE may also have clinical relevance since chronic neurodegenerative diseases such as Alzheimer's disease (AD) involve inflammation and oxidative stress [15–19]. Furthermore, Roses [5] and colleagues [20,21] have demonstrated an increased susceptibility to AD in individuals expressing the APOE4 allele.

Using a macrophage cell line (RAW 264.7) we have more fully examined the specific factors that regulate the effect of apoE on NO production. RAW 264.7 cells have been well characterized as a model for macrophage function and share functional homology with other tissue macrophages including the CNS macrophage, the microglia [13,22,23]. Thus, these cells can be used as a model for microglia. Our new data demonstrate a novel action of apoE on the regulation of macrophage function and the immune response.

2. Methods

2.1. Materials

Delipidated apoE purified from human serum was obtained from Chemicon International (Temcula, CA) or ICN Pharmaceuticals (Costa Mesa, CA) and used immediately on dilution. Since mixed human serum was the source of this apoE and since approx. 84% of the population carries the APOE 3 allele [24], it is likely that apoE3 is the predominant isoform in this source. Recombinant human apoE3 purchased from commercial sources was found to be unstable in our cultures and was not used in this study. Human apoE3 was also isolated from serum-free conditioned medium (CM) of primary astrocyte cultures of mice made transgenic for human apoE3 on a mouse apoE knockout background and was a generous gift from Dr. David Holtzman and Dr. Ron DeMattos (Washington University School of Medicine, St. Louis, MO). Conditioned medium (3 days) from the transgenic apoE3 or apoE4 astrocyte cultures was sterile filtered and circulated over a monoclonal antibody column at 4°C for 16 h. After washing with 200 ml PBS, apoE was eluted with 3 M sodium thiocyanate and dialyzed extensively against PBS containing 0.01% azide. The particles were then characterized by apoE ELISA, Western, Coomassie, and non-denaturing gradient gels. The fully characterized stock solution was diluted approx. 1/1000 into normal medium, thus the azide concentration was considered to be negligible. Delipidated apolipoprotein B purified from human serum was purchased from Chemicon. Polyinosinic acid:polycytidylic acid (PIC) (K^+ salt) was purchased from Sigma (St. Louis, MO). Lipopolysaccharide (LPS; O55:B5) was obtained from Calbiochem (San Diego, CA) and recombinant murine interferon-γ (IFNγ) was obtained from Gibco (Rockville, MD). The inducible nitric oxide synthase (iNOS) antibody (#06-295) was purchased from Upstate Biotechnology (Lake Placid, NY). Normal tissue culture medium and supplements were purchased from Gibco while arginine-free DMEM was purchased from ICN.

2.2. Cultures

The mouse macrophage cell line (RAW 264.7) was obtained from ATCC (Reston, VA) and cultured in DMEM containing 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 100 U/ml pen-strep and 15 mM HEPES buffer. Cells were grown in a humidified 95% air, 5% oxygen atmosphere at 37°C until confluent. Prior to the experiment, the cells were removed from the flask by trypsinization, spun at $1000 \times g$ to pellet and resuspended in growth medium. The cells were plated into the center wells of 96-well dishes at an initial density of 40 000 cells per well and allowed to recover overnight. In some cases, an increased number of cells were plated into six-well dishes for PCR or Western blot analysis.

2.3. NO assay

Nitric oxide production was measured from the supernatant levels of nitrite, the stable biological oxidation product of NO, using the Griess reaction as described previously [25,26] or a Seivers nitric oxide analyzer (NOA 280, Seivers Instrument, Boulder, CO) which detects the chemiluminescent signal generated by the reaction of nitric oxide with ozone. RAW cells plated as described above were treated with 100 U/ml recombinant murine IFNγ in normal growth medium (IFNγ-primed) or remained untreated (non-primed). After 8–12 h of pretreatment the medium was replaced with serum-free medium containing the experimental agents for an additional 4–24 h. Cell supernatants were then collected and the nitrite levels determined using sodium nitrite as the standard. The protein content of the wells was obtained using the Pierce BCA assay (Rockford, IL) with bovine serum albumin as the standard. Data are presented as μmoles of nitrite/40 000 cells or as μmoles of nitrite/μg protein. Two to six wells of cells were assayed per experimental condition for at least three different culture groups. Statistical analysis was done using an ANOVA with the Bonferroni correction or using the unpaired Student's *t*-test.

2.4. Arginine uptake

RAW cells were plated into 96-well plates (approx. 8×10^4 cells per well) and allowed to adhere overnight. Cells were then primed with IFNγ (100 U/ml) for 8–12 h in cell culture medium. After the pretreatment, the culture medium was aspirated and discarded. The cells were then treated for an additional 4 h with fresh serum-free medium containing 10 μM arginine plus the various treatment conditions. This value of arginine was chosen because it more closely represents extracellular arginine levels in the CNS [27]. After the 4 h, medium was either discarded or saved for nitrite analysis. The cells were washed with warm (37°C) uptake buffer containing in mM: NaCl, 137; KCl, 2.7; $CaCl_2$, 1.5; KH_2PO_4, 1.2; $MgSO_4$, 1.0; HEPES, 20; adjusted to pH 7.4. L-[^{3}H]Arginine (1 Ci/ml, New England Nuclear) uptake was measured by incubating the cell monolayers for 5 min at 37°C in the presence of increasing concentrations of unlabeled L-arginine (1–100 μM). After the 5 min incubation, the plate was inverted to remove any buffer containing excess tracer, and placed on a tray of melting ice. Ice-cold buffer containing 10 mM unlabeled substrate was then added (0.1 ml). The buffer was removed by inversion of the plate and blotting on a paper towel. The washing procedure was repeated twice more for a total of three washes. Radioactivity in 1.0% Triton X-100 extracts was then determined by liquid scintillation counting. Non-specific uptake of L-[^{3}H]arginine was found by repeating the above procedures at 4°C. Values of non-specific uptake at 4°C were subtracted from the values at 37°C. A portion of the cell digest was used for protein measurement (Pierce BCA assay). Uptake (cpm/mg protein) was then calculated and data are expressed as the average percent change from the non-primed, untreated control. Statistical analysis was done using an unpaired Student's *t*-test.

2.5. Western blot analysis

Cell lysates from treated and untreated RAW cells were analyzed by Western blot for immunoreactivity to iNOS. Protein concentration of each cell lysate sample was determined using the Pierce BCA assay. Sample buffer was then added to equal protein concentrations from the cell samples and the mixtures boiled for 5 min. These samples were loaded onto pre-cast gradient gels, electrophoresed for 1 h at constant voltage and then transferred to nitrocellulose for 1 h. After blocking (Blotto), the primary antibody was applied overnight at 4°C followed by washing in three changes of PBS and then a single wash of Blotto. The appropriate biotinylated secondary antibody was applied for 1 h, the blot washed and then incubated with extravidin peroxidase. Location of immunoreactive bands was detected by enhanced chemiluminescence (ECL) (Amersham, Newark, NJ) per manufacturer's instructions. Biotinylated molecular weight standards were used to identify the approximate size of the immunoreactive bands for each Western blot.

2.6. RT-PCR

Total RNA was prepared from treated or untreated RAW cells using the TRizol reagent (Gibco) according to the manufacturer's protocol. Total

RNA was reversed-transcribed using Thermoscript RT (Gibco) with specific antisense oligonucleotides TTATTCTGGGTCTCAGGGGCTGAAG and CGCTTTATCTTCTGGCTGCTGGTC (for mCAT1), GCGTTTGAGTATGTCAGATGGGGC and AGGCATCCTCATCGTCTTCTTCGTC (for mCAT2) and GACCATGAGTGAATCCAACGGCAC and TACGGCAAGCAGTAGGGAGCAAAC (for mCAT3). Conditions for PCR amplification of the resulting first-strand DNA template were 95°C (denaturing), 64°C (annealing), 72°C (extension) for 25 cycles using platinum *Taq* DNA polymerase (5 U/ml) and 1.8 mM $MgSO_4$. PCR products were sequenced to confirm their identity.

Relative values for mRNA expression were obtained by densitometry (NIH Image) of PCR products separated on polyacrylamide gradient gels, stained with ethidium bromide and viewed under ultraviolet light. The ratios of experimental bands to corresponding control (β-actin) bands were used as a relative measurement of gene expression. For each gene, ratiometric data for each triplicate group per

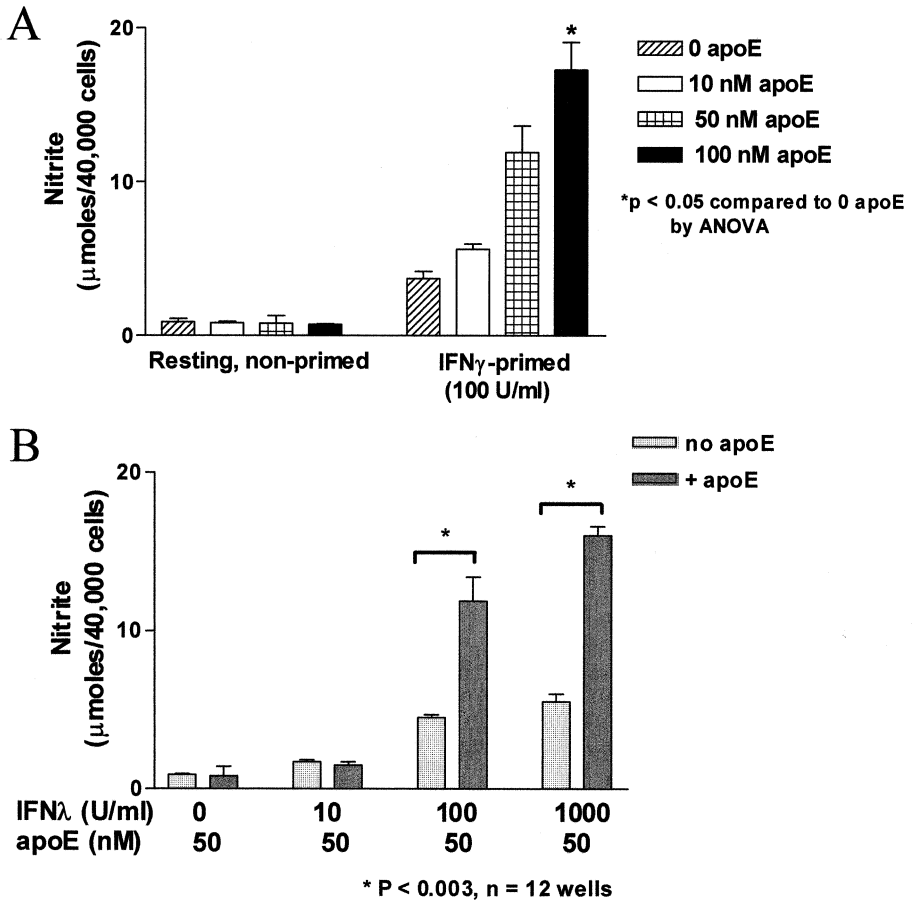

Fig. 1. (A) The effect of apoE on non-primed (resting) and interferon-γ (IFNγ)-primed RAW cells. RAW cells were either pretreated with 100 U/ml recombinant murine IFNγ for 12 h in normal growth medium or remained untreated. The medium was then replaced with serum-free medium containing varying concentrations of apoE (10–100 nM) for an additional 24 h. Data points represent the average ± S.E.M. supernatant nitrite value (μmoles/40 000 cells) for a minimum of three wells for four different culture groups. *$P < 0.05$ level compared to IFNγ priming only (ANOVA). (B) ApoE-stimulated production of NO is increased with increasing doses of IFNγ. RAW cells were pretreated with growth medium containing 10–1000 U/ml IFNγ for 12 h or remained untreated. The medium was then replaced with serum-free medium containing 50 nM apoE and supernatant nitrite levels were measured at 24 h. Data points represent the average ± S.E.M. nitrite value (μmoles/40 000 cells). n = at least 12 wells assayed per experimental condition. *$P < 0.003$ compared to IFNγ alone.

experimental condition were averaged. Statistical differences were determined using an unpaired Student's *t*-test.

3. Results

RAW 264.7 cells, a mouse macrophage clonal cell line, generate NO when stimulated by factors associated with tissue injury and inflammation. To determine the effect of apoE on the production of NO by RAW cells, we treated non-primed (resting) and IFNγ-primed cells with apoE. The resultant NO production was determined by detecting nitrite concentrations in the cell supernatants. As shown in Fig. 1A, apoE had no effect on NO production in resting (non-primed) RAW cells. However, when RAW cells were primed for 12 h with recombinant murine IFNγ and then treated with apoE, NO production was increased compared to non-primed conditions. ApoE increased NO production in a dose-dependent fashion up to 100 nM, the maximal apoE concentration studied (Fig. 1A). In addition, the level of nitrite produced by apoE was increased with increasing

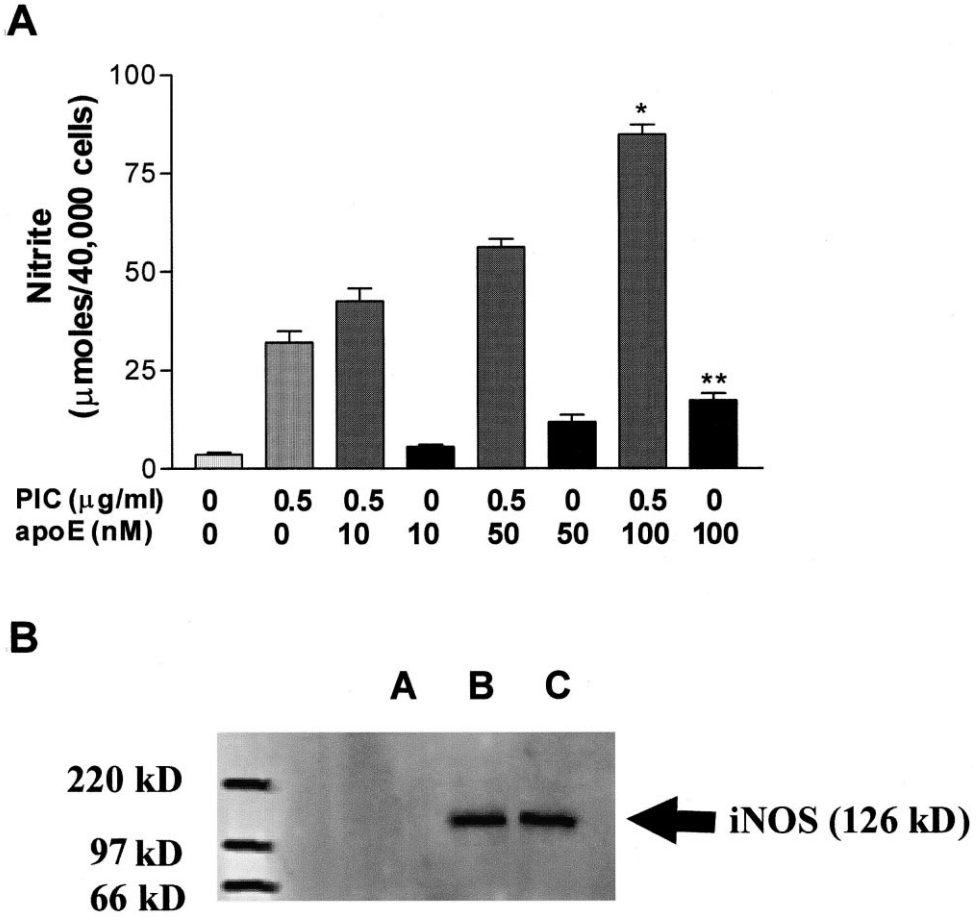

Fig. 2. (A) ApoE enhanced the effect of polyinosinic acid:polycytidylic acid (PIC), a double stranded polyribonucleotide, on NO production. RAW cells were pretreated with 100 U/ml IFNγ for 12 h, followed by treatment for 24 h with serum-free medium containing apoE alone (10–100 nM), PIC alone (0.5 μg/ml) or PIC (0.5 μg/ml) plus apoE (10–100 nM). Data points represent the average ± S.E.M. supernatant nitrite value (μmoles/40 000 cells). $n = 12$ wells assayed. $^{*}P < 0.001$ compared to PIC by ANOVA. $^{**}P < 0.05$ compared to IFNγ priming alone by ANOVA. (B) Detection of immunoreactive iNOS in IFNγ-primed RAW cells treated with PIC (0.5 μg/ml) alone and in combination with apoE (50 nM) for 12 h. Representative Western blot from three separate experiments. Lanes: A, non-primed cells; B, IFNγ primed+PIC; C, IFNγ primed+PIC+apoE. Molecular weight standards from the same gel are also shown.

concentrations of IFNγ used in the priming process (Fig. 1B). Enhanced production of NO was also seen in RAW cells primed with IFNα in place of IFNγ (data not shown), indicating that both lymphokines could promote the effect of apoE.

To determine if apoE modulated NO production in the presence of agents known to upregulate the expression of inducible nitric oxide synthase (iNOS), the enzyme that catalyzes the production of NO from arginine, we examined the effect of apoE on NO production in RAW cells treated with PIC or LPS. Both PIC, a double stranded polyribonucleotide which serves as a viral mimetic, or LPS, a bacterial coat component, are well known to induce iNOS mRNA and protein expression and initiate NO production in interferon-primed macrophages [12,28,29]. Consistent with the ability of PIC to induce iNOS mRNA and protein, treatment of IFNγ-primed RAW cells with PIC (0.5 μg/ml) for 24 h resulted in a significant increase in supernatant nitrite levels compared to cells primed with IFNγ alone (Fig. 2). The simultaneous addition of apoE and PIC, however, significantly increased NO levels compared to PIC or to apoE alone. This effect of apoE was dose-dependent and could not be explained by a simple additive effect of PIC and apoE on supernatant nitrite levels at each concentration of apoE. Immunodetection (Western blot) of iNOS protein expression was performed on cell lysates from RAW cells treated with a non-maximal dose of PIC (0.5 μg/ml) in the presence and absence of 50 nM apoE. A representative blot is shown in Fig. 2B. No apparent change in iNOS protein expression was seen when PIC-treated cells were simultaneously treated with apoE.

Enhancement of NO production by apoE was not specific to PIC since LPS-induced NO production was also increased. Simultaneous exposure of IFNγ-primed RAW cells to LPS (0.1 μg/ml) and apoE (50 nM) increased supernatant nitrite levels compared to LPS alone (Fig. 3). Treatment of the RAW cells with apolipoprotein B (10–100 nM), another form of circulating lipoprotein, or with boiled apoE did not increase supernatant nitrite levels.

Since apoE from plasma differs from apoE derived from the CNS [30], we tested the effect of apoE isolated from astrocytes, the primary source of apoE in the CNS [31]. CM from primary astrocyte

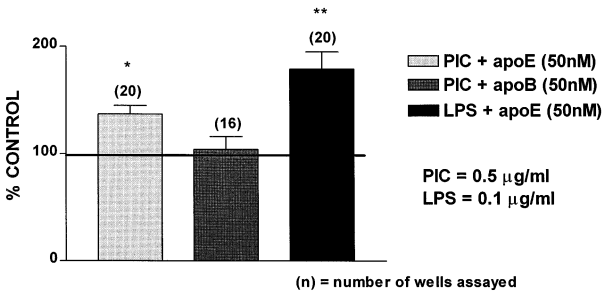

Fig. 3. Comparison of NO production. IFNγ-primed RAW cells were treated for 24 h with PIC (0.5 μg/ml) plus apoE (50 nM) isolated from human plasma, with PIC (0.5 μg/ml) plus apolipoprotein B (50 nM) isolated from human plasma or with LPS (0.1 μg/ml) plus 50 nM apoE. Data points represent the average percent of control ± S.E.M. obtained from 100× the ratio of the experimental nitrite level to the nitrite level in PIC (or LPS)-treated cells (PIC or LPS alone = 100%). (*n*) = number of wells assayed. $*P < 0.02$; $**P < 0.001$.

cultures of human apoE3 transgenic mice was used as a source of CNS-derived apoE3 [30,32,33]. The apoE produced by these astrocytes has been fully characterized and is comprised of lipid containing particles similar to those isolated from cerebral spinal fluid (CSF) [33]. RAW cells were pretreated with 100 U/ml IFNγ for 12 h and then exposed for an additional 24 h to 0.5 μg/ml PIPC alone or in the presence of 50 nM apoE3 purified from astrocyte CM (Fig. 3). Supernatant nitrite levels were significantly ($P < 0.05$) increased in the presence of astrocyte CM-apoE3 plus PIPC (3.3 ± 0.43 μmoles) compared to PIC alone (2.1 ± 0.2 μmoles), suggesting that apoE isolated from the conditioned medium of astrocytes has an effect similar to circulating apoE.

The synergistic effect of apoE on PIC-induced NO production, the ability of apoE to interact with two distinct types of inflammatory agents (LPS and PIC) and the apparent lack of an effect of apoE on iNOS protein expression suggested that apoE could potentially regulate multiple components of the NO biosynthetic pathway. One alternate component is arginine (substrate) availability. Activity of iNOS, NO production and macrophage cytotoxicity are tightly regulated by extracellular arginine availability [9,34–37]. To determine if apoE altered the relationship between extracellular arginine concentration and NO production and thus, altered the availability of substrate for iNOS enzymatic activity, we measured NO production at varying concentrations of extra-

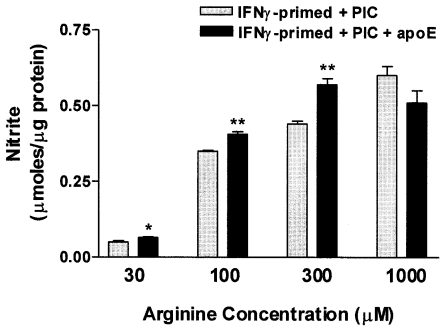

Fig. 4. The effect of extracellular arginine concentration on NO production. IFNγ-primed RAW cells were treated for an additional 8 h in medium containing various concentrations of arginine plus 0.5 μg/ml PIC or 0.5 μg/ml PIC plus 50 nM apoE. Data points represent the average level of supernatant nitrite/μg protein ±S.E.M. (n) = five wells assayed. *$P < 0.01$ compared to PIC alone; **$P < 0.001$ compared to PIC alone.

cellular arginine in IFNγ-primed RAW cells treated with PIC alone (0.5 μg/ml) and in combination with apoE (50 nM). As shown in Fig. 4, supernatant nitrite levels increased with increasing extracellular arginine concentration under both treatment conditions, reaching a maximum at approx. 1 mM arginine. Treatment with apoE plus PIC, however, significantly increased nitrite levels at submaximal arginine concentrations compared to PIC alone.

One way to modify the relationship between extracellular arginine levels and NO production is to increase the uptake of arginine across the plasma membrane and into the intracellular compartment. Arginine uptake across the plasma membrane in macrophages is dependent on the activity of system y^+ transporters, also known as cationic amino acid transporters (CAT) [36,38–41]. To determine if apoE altered arginine transport across the plasma membrane in RAW cells, we examined the effect of apoE on [^{3}H]arginine uptake. For these experiments, cells were pretreated with 100 U/ml IFNγ in normal medium for 8 h and then equilibrated for an additional 4 h in medium containing varying concentrations of arginine (10–100 μM) in the presence of apoE alone, PIC alone or apoE plus PIC. Arginine uptake across the plasma membrane was then determined for each of the experimental conditions over a range of extracellular arginine concentrations. As shown in Fig. 5, IFNγ priming increased arginine uptake compared to non-primed, untreated RAW cells. Treatment of IFNγ-primed cells with PIC further enhanced arginine uptake. However, simultaneous treatment with PIC and apoE produced the greatest increase in arginine transport. No significant change in arginine uptake was observed in IFNγ-primed cells treated with apoE alone (Fig. 5) or in primed cells treated with apolipoprotein B (apoB) plus PIC (data not shown). Average values for the apparent V_{max} and the affinity constant, K', under the above conditions are shown in Table 1. Treatment with PIC plus apoE significantly increased the

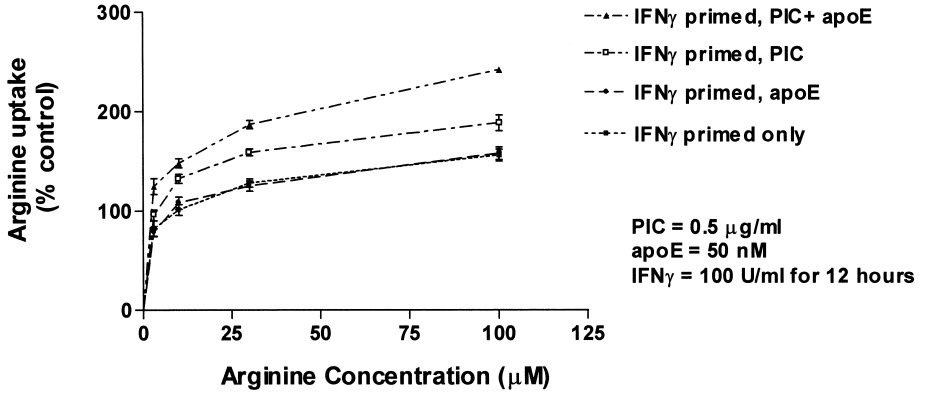

Fig. 5. Arginine uptake across the plasma membrane in apoE-treated RAW cells. RAW cells were primed with IFNγ for 12 h or remained untreated. These cells were then treated for an additional 4 h with medium containing varying concentrations of arginine in the presence or absence of 50 nM apoE, 0.5 μg/ml PIC or 50 nM apoE plus 0.5 μg/ml PIC. $^3H^+$-Arginine uptake was then determined as described in Section 2. Data are presented as percent of control uptake (average ± S.E.M., n = 11–16 wells assayed per experimental condition) and were determined from the ratio of the specific arginine uptake (cpm/mg protein) under experimental conditions to the specific arginine uptake (cpm/mg protein) under non-primed (resting) conditions (= 100%). $P < 0.001$ for PIC-treated compared to IFNγ-primed only and $P < 0.02$ for PIC+apoE-treated compared to PIC-treated conditions using ANOVA.

Table 1
Changes in arginine transport maximum and affinity

	IFNγ-primed untreated	IFNγ-primed apoE	IFNγ-primed PIC	IFNγ-primed apoE+PIC
V_{max}	95.7 ± 15	118.7 ± 25.4	110.9 ± 2.5*	144.0 ± 5.6**
K'	71.9 ± 22	105 ± 38.5	25.3 ± 1.6*	19 ± 2.1**

Average values were determined from a non-linear regression analysis on non-transformed data (Graph Pad Prism). (n) = five separate experiments with five wells per experiment. *$P < 0.02$ compared to IFNγ-primed, apoE+PIC; **$P < 0.04$ compared to IFNγ-primed, untreated.

maximal arginine transport and lowered K' compared to IFNγ-primed untreated cells or compared to IFNγ-primed, PIC-treated cells. Thus, our data demonstrate that apoE in combination with PIC induces a change in both maximal transport and transporter affinity for arginine.

Using semiquantitative RT-PCR [42,43], we also determined the expression of mRNA for CAT1, CAT2 and CAT3 in non-primed (resting) RAW cells and in RAW cells treated as described above. Under non-primed conditions, mRNA was detected for CAT1 (Fig. 6A,B) but not for CAT2 (Fig. 6A,C). IFNγ-primed cells, IFNγ-primed cells treated with apoE or IFNγ-primed cells treated with PIC dis-

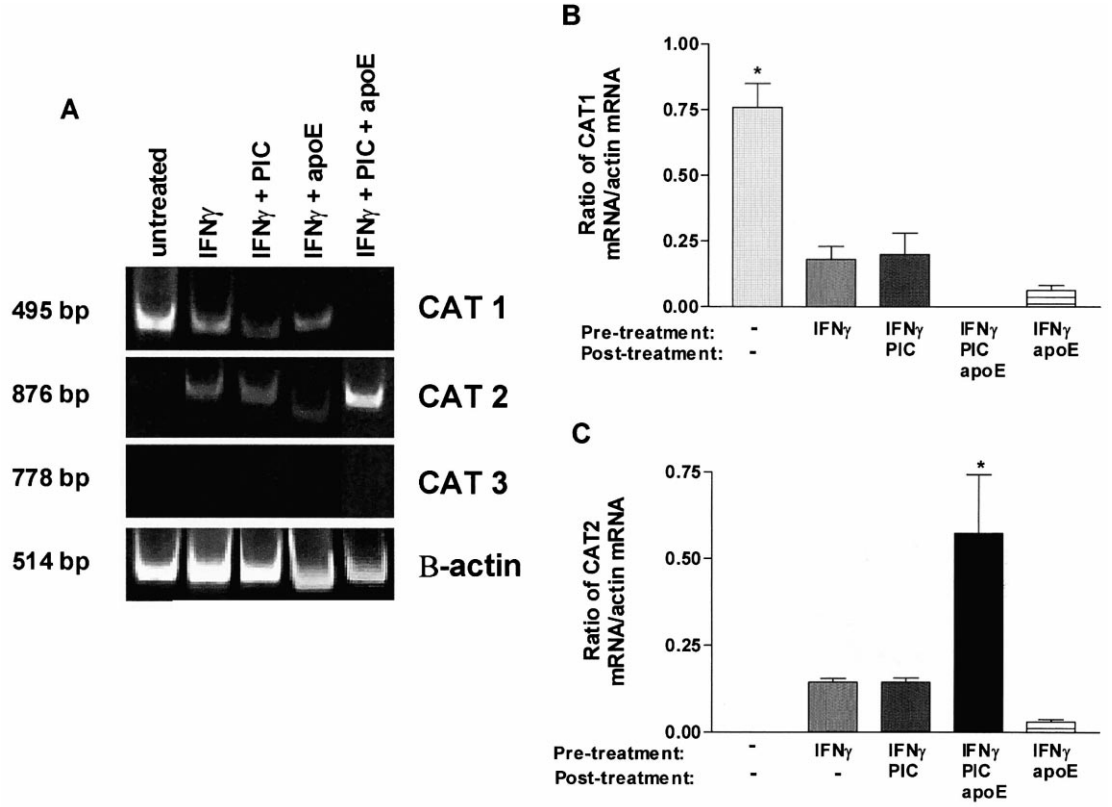

Fig. 6. (A) Expression of CAT transporter mRNA in RAW cells treated with apoE. RAW cells were non-primed or primed for 8 h with IFNγ (100 U/ml) and then treated for 4 h with PIC (0.5 μg/ml), PIC plus apoE (50 nM) or apoE alone (50 nM). Semiquantitative RT-PCR was used to probe expression of the mRNAs for CAT1, CAT2 or CAT 3. Data are representative for one out of three separate groups of RAW cells. (B) Relative expression of CAT1 mRNA. Densitometry was used to determine the average density of each mRNA band for each experimental condition. The data represent the average ratio of the density (± S.E.M., $n = 3$) for CAT1 mRNA to the density for β-actin mRNA in the same sample. *$P < 0.01$ compared to other treatment conditions. (C) Relative expression for CAT2 mRNA. The data represent the average ratio of the density for CAT2 mRNA to β-actin mRNA for each experimental condition. n = three separate cell groups. *$P < 0.03$ compared to other treatment conditions.

played measurable levels of CAT1 and CAT2 mRNA (Fig. 6A–C). However, simultaneous treatment with PIC and apoE significantly increased the relative expression of CAT2 mRNA while apparently reducing the levels of CAT1 mRNA (Fig. 6A–C). A signal corresponding to CAT3 mRNA expression was not observed in RAW cells under any of the treatment conditions.

4. Discussion

We previously reported that exogenous application of apoE to human MDM induced a significant increase in NO production [8]. This effect has now been observed in human microglia, rodent microglia, murine peritoneal macrophages and murine macrophage clonal cells lines such as the RAW cells used here [44]. Our new data further support a role for apoE in the innate immune response mediated by macrophages and particularly in the regulation of nitric oxide production. For example, the ability of apoE to increase NO production in RAW cells is tightly linked to lymphokine-mediated (IFNγ or IFNα) activation. Both IFNγ and IFNα increase the functional responses of macrophages and frequently act in concert with other cytoactive factors to induce tissue defense mechanisms such as NO production [12,22,29]. IFNγ priming facilitates the action of apoE on NO production in RAW cells. ApoE also works in concert with other macrophage activating agents like LPS, a bacterial coat component, or PIC, a viral mimetic, which are well known to induce iNOS mRNA and protein expression, iNOS enzymatic activities and to increase NO levels [9,12,13,29,45,46]. We now show that ApoE potentiated the action of either LPS or PIC in a dose-dependent fashion. The effect is specific to apoE since treatment of the RAW cells with another apolipoprotein, apoB, or with heat-inactivated (boiled) apoE did not alter supernatant nitrite levels from non-lipoprotein-treated controls.

We also examined the effect of apoE isolated from astrocyte CM on NO production. Astrocytes are the primary source of apoE in the CNS [31,32,47] and release a form of lipoprotein which differs in lipid content and structure compared to circulating apoE [30]. Sun et al. [33] have characterized apoE isolated from primary cultures of astrocytes derived from mice made transgenic for human APOE3 on a mouse APOE null background. Treatment of RAW cells with this form of apoE3 produced a similar effect as the apoE purified from human plasma, suggesting that the lipoprotein site which interacts with the macrophage is conserved in both forms and that lipid content is not a critical limiting factor.

ApoE-induced modulation of NO production can occur at several points in the biosynthetic pathway. The most common mechanism for regulation of macrophage NO is via the induction of the iNOS gene and the consequent expression of iNOS mRNA and protein [9,11,46,48]. Consistent with previously published findings [14,28,46,49], our data demonstrate that treatment with PIC induces iNOS protein expression in macrophages. However, the apparent lack of an effect of apoE on iNOS protein expression in PIC-treated cells was surprising and suggested the possibility of alternate mechanisms for regulation of NO. Further support for alternate sites of action was provided by other data. For example, the level of nitrite produced by the combination of PIC and apoE is greater than that level expected from an additive effect of PIC and apoE alone. Furthermore, the combination of apoE and PIC increased NO production in the RAW cells at submaximal arginine concentrations compared to PIC alone. The resultant shift in the arginine-NO curve is similar to those changes seen when arginase, an intracellular enzyme that competes with iNOS for arginine, is inhibited [35]. While this does not rule out a synergistic effect of apoE and PIC on induction of iNOS mRNA and protein, our data are consistent with increased substrate (arginine) availability for iNOS and hence, for NO production. Finally, apoE has been shown to affect NO production in cells that contain cNOS instead of iNOS. Riddell et al. [50] recently demonstrated that inhibition of platelet aggregation by apoE is mediated by NO. The similar overall effect of apoE on disparate cell types and NOS subtypes further suggests a potential novel site of action for apoE.

Arginine transport across the plasma membrane was considered a likely candidate for modulation by apoE since arginine availability is rate limiting for NO production [9,34,36,51,52]. Macrophage arginine uptake is mediated by a family of Na^+-inde-

pendent, temperature-sensitive cationic transporters known as the system y^+ transporters or, more recently, as the CAT transporters [36,40,53]. Of the three subtypes of CAT proteins, CAT2 is associated with activated macrophages, including RAW cells, and is induced by LPS or other inflammatory mediators [36,39,40,53–55]. Kakuda et al. [53] and Stevens et al. [56] have further demonstrated that CAT2 expression is linked with iNOS mRNA and protein expression and NO production, suggesting that this transporter is a major factor in the pathway for NO synthesis during the innate immune response. Consistent with these previously published findings [40,53,56], we have demonstrated the presence of an arginine uptake process with characteristics of system y^+ transporters in RAW cells. Our data further demonstrate that viral mimics such as PIC increase transport of arginine in IFNγ-primed cells and that apoE enhances this process compared to PIC alone. This effect was seen over a physiological range of arginine concentration, is within the concentration range of apoE found in the CNS [6] and was specific for apoE since treatment with PIC plus apoB did not produce a similar increase in arginine transport.

ApoE's action on arginine transport may be explained, in part, by a change in the relative expression of CAT2 transports. Kakuda et al. [53,57] observed a decrease in mRNA for CAT1 concomitant with an increase in mRNA expression for CAT2 in LPS-activated macrophages. We observed a similar change in mRNA for CAT1 and CAT2 in RAW cells primed with IFNγ alone, or in IFNγ-primed cells treated with PIC or apoE alone. The combination of PIC and apoE, however, was associated with a large, relative increase in mRNA for CAT2 while CAT1 mRNA levels were undetectable under the same conditions. It is not clear how this dramatic switch in mRNA from CAT1 to CAT2 translates to a change in transport protein expression or to a change in NO production. Kinetic analysis of these transporters has shown a remarkable similarity between the two subtypes although small differences in apparent V_{max} and arginine affinity are observed [40]. Regardless, this type of change is clearly part of the response of a macrophage to inflammation and injury and opens new avenues for further investigation.

The modulation of macrophage arginine transport and, consequently, of NO production by apoE is a novel regulatory mechanism for an apolipoprotein. Our data suggest that the interaction between CAT transporters, NO and apoE may underlie a basic component of the tissue response to injury. This interrelationship may be of importance in disease processes such as chronic neurodegeneration where immune activation, oxidative stress and apolipoprotein E are known to play a role [18,58–61].

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