

# Acetate Intolerance Is Mediated by Enhanced Synthesis of Nitric Oxide by Endothelial Cells

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**Abstract.** The clinical picture of acetate intolerance strictly mimics the nitric oxide (NO) effect, including smooth muscle relaxation and extreme vasodilation. Because acetate induces production of cAMP, which is a powerful stimulus of NO synthase (NOS), we evaluated the effect of different dialysate solutions with and without acetate on NOS activity in endothelial cells (EC). NOS activity of EC, evaluated as H3-citrulline produced from H3-arginine, was modulated by the dialysate composition (*e.g.*, 38 mmol/L acetate produced an increase of  $3.2 \pm 0.39$ -fold compared with basal values ( $P < 0.0005$ ), and the small amount of acetate (4 mmol/L) in 35 mmol/L bicarbonate solution increased the NOS activity by  $2 \pm 0.49$ -fold ( $P < 0.05$ ). Conversely, the acetate-free solution produced no effect on NOS activity. The mRNA encoding for

inducible NOS was highly expressed in EC incubated with acetate buffer and also with acetate in bicarbonate dialysis buffer. The EC proliferative index was depressed by acetate ( $P < 0.0005$ ), and tumor necrosis factor synthesis was increased ( $P < 0.0005$ ) compared with acetate-free buffer. This study suggests that dialytic "acetate intolerance" can be induced by the activation, through cAMP and tumor necrosis factor release, of NOS. The small amount of acetate in bicarbonate dialysate, although capable of inducing *in vitro* NOS activation, is likely to be rapidly metabolized, whereas the large amounts of this anion in acetate fluids overwhelm metabolism by the liver. Acetate-free dialysate is the only solution that provides an acceptable level of biocompatibility both *in vivo* and *in vitro*. (J Am Soc Nephrol 8: 1431–1436, 1997)

Several studies have shown the beneficial effects of bicarbonate instead of acetate-containing dialysates in reducing the morbidity associated with dialysis (1). The benefits include a lower incidence of arterial hypotension (1–5), improved left ventricular function (2), and decreased incidence of nausea, vomiting, and headache (1). These symptoms are the hallmarks of so-called "acetate intolerance." Acetate buffer as a dialysis fluid induces hemodynamic instability through peripheral vasodilation and depression of myocardial contractility (2,5). The mechanisms by which acetate induces these effects are still not understood.

It has been proposed that acetate induces vasodilation by deranging AMP metabolism. Acetate infusion (6) produces an increase in intracellular levels of AMP as a result of conjugation of acetate with coenzyme A (CoA) to form acetyl-CoA. This reaction consumes ATP with production of adenosine and AMP, which can be transformed in cAMP. In fact, ATP, AMP, and ADP are interconvertible through adenylate kinase. ATP is the substrate of adenylate cyclase, an integral membrane protein, with final production of cAMP.

Indeed, during acetate-buffered hemodialysis, platelets become depleted of ATP (7). Most of the adenine nucleotides can act as vasodilators in precontracted vessel strips, possibly by virtue of their effect on purinergic receptors (8), which also have recently been found on endothelial cells (EC). AMP, but not adenosine, in high concentrations relaxes the vasopressin-constricted rat tail artery (9). Studies on healthy volunteers showed that elevation in blood acetate from the physiological level of 0.1 to 1 mM resulted in increased heart rate and decreased diastolic BP (10). Moreover, acetate *per se* can modulate some cellular activities, including interleukin (IL)-1 synthesis (11). However, the exact mechanism of vasorelaxation induced by acetate metabolism is not yet defined.

cAMP and cytokines (12,13) are powerful stimuli of nitric oxide (NO) synthase (NOS) activity, an NADPH-dependent family of enzymes. NOS transforms L-arginine (L-Arg) to L-citrulline and NO with a 1:1 stoichiometry (14,15). NO is a powerful mediator. Smooth muscle relaxation is one of the most well-known activities induced by NO, and this effect is mediated by a dose-dependent increase in the levels of cyclic guanosine monophosphate in smooth muscle (16,17) consequent to the activation of soluble guanylate cyclase.

Because some effects of NO overlap with those induced by acetate, and acetate induces production of cAMP and cytokines, which in turn can give rise to NO synthesis, we focused on whether the acetate contained in dialysis fluids can influence NO production by EC. We also evaluated the influence of acetate on tumor necrosis factor (TNF)- $\alpha$  production and proliferative activity of EC.

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## Materials and Methods

### Experimental Design

Murine EC were incubated for 6 h with different dialysis fluids with or without acetate: (1) acetate, 38 mmol/L; (2) bicarbonate, 35 mmol/L, acetate 4 mmol/L; (3) acetate-free buffer (AFB). All of these conditions were studied with or without lipopolysaccharide (LPS) (10  $\mu$ g/ml). Cell culture supernatants, cell enzymatic and proliferative activity, and specific mRNA synthesis were then evaluated.

### Cells

To avoid using different human cell donors, sequential passages, and protracted manipulations, the murine endothelioma cell line T End.1 (18) was selected for the experiments. T End.1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO) containing 10% fetal calf serum (Sigma), penicillin, streptomycin, and amphotericin (Sigma). Experiments were performed at the 98th through 166th passage. The mouse EC line End.1, derived from a thymic hemangioma expressing the polyoma middle T antigen (19), retains functional properties of normal EC. These cells proliferate at confluence without overgrowth, take up acetylated LDL, and express CD31, VCAM-1, E-selectin, and P-selectin, respond to IL-1 $\beta$ , TNF- $\alpha$ , and specific EC growth factors, and produce IL-6 and chemokines (19,20).

### Culture Conditions

EC were cultured at 37°C in an environment of 95% air and 5% CO<sub>2</sub> and in DMEM-supplemented media. Cells were then incubated for 6 h with different dialysis fluids with or without acetate: (1) 38 mmol/L acetate, 140 mmol/L Na<sup>+</sup>, 2 mmol/L K<sup>+</sup>, 1.75 mmol/L Ca<sup>2+</sup>, 0.75 mmol/L Mg<sup>2+</sup>, 110.0 mmol/L Cl<sup>-</sup>, and 5.55 mmol/L glucose; (2) 35 mmol/L bicarbonate, 4 mmol/L acetate, 143 mmol/L Na<sup>+</sup>, 2 mmol/L K<sup>+</sup>, 1.75 mmol/L Ca<sup>2+</sup>, 0.75 mmol/L Mg<sup>2+</sup>, 110.5 mmol/L Cl<sup>-</sup>, and 5.55 mmol/L glucose; (3) AFB: 139 mmol/L Na<sup>+</sup>, 2 mmol/L K<sup>+</sup>, 2 mmol/L Ca<sup>2+</sup>, 0.37 mmol/L Mg<sup>2+</sup>, 145.74 mmol/L Cl<sup>-</sup>, and 5.5 mmol/L glucose. At the end of incubation, culture supernatants were collected for TNF- $\alpha$  measurements. After three washes with Hanks' Ca<sup>++</sup>, Mg<sup>++</sup>-free solution (Sigma), EC were trypsinized and suspended in reaction buffer for NOS activity (see below) or in sterile phosphate-buffered saline, 0.15 M (pH 7.3), for RNA extraction. The cells were snap-frozen and stored at -80°C until use.

### NO Measurement

EC were frozen in 1 ml of reaction buffer containing (in mM) 20 Hepes, 0.5 ethylene diamine tetra-acetic acid, and 1 dithiothreitol, pH 7.2, sonicated on ice with three 10-s bursts. In each test tube, the following reagents were added to 100  $\mu$ l of sonicate at the final concentrations: 2 mM NADPH, 1.5 mM CaCl<sub>2</sub>, 1 to 100  $\mu$ M L-Arg, 2.5  $\mu$ Ci (= 0.4  $\mu$ M) and L-[2, 3, 4, 5-<sup>3</sup>H]arginine monohydrochloride (62 Ci/mmol; Amersham International, Bucks, United Kingdom). After a 30-min incubation at 37°C, the reaction was stopped by adding 2 ml of 20 mM Hepes-Na, pH 6.0, containing 2 mM ethylenediamine tetra-acetic acid. The whole reaction mixture was applied to 2-ml columns of Dowex AG50WX-8 (Na<sup>+</sup> form; Aldrich, Milano, Italy) and eluted with 4 ml of water. At pH 6.0, arginine is negatively charged, whereas citrulline is neutral; the Dowex resin is a cationic exchanger that binds arginine but not citrulline in these conditions. The radioactivity corresponding to [<sup>3</sup>H]citrulline content in the 6-ml eluate was measured by liquid scintillation counting. NOS activity was expressed as picomoles of citrulline generated per minute of incubation per milligram of EC protein. The protein content of EC was assessed with the modified micro-Lowry method (kit from

Sigma). T End.1 cells have a detectable basal NOS activity (21). However, NOS activity of EC incubated with various dialysis fluids was expressed in our experiments as fold increase compared with values obtained in cells incubated with DMEM.

### RNA Extraction and Reverse Transcription-PCR Amplification of mRNA for Inducible NOS

Total RNA was extracted from EC and snap-frozen in liquid nitrogen, using the RNeasy (Qiagen, Crawley, UK) method, following the manufacturer's instructions. EC were suspended in RNeasy lysis buffer at the proportion of 10<sup>6</sup> cells/ml. The suspension was vortexed for 15 s and incubated in chloroform at 4°C for 15 min. After 12,000  $\times$  g centrifugation at 4°C for 15 min, the supernatant was extracted with an equal volume of isopropanol. After another incubation at -20°C for 45 min and 12,000  $\times$  g centrifugation for 15 min, the pellet was washed twice in 75% ethanol and resuspended in double-distilled water. The concentration and purity of RNA were determined by absorbance at 260 and 280 nm.

Reverse transcription of total RNA to cDNA was performed using the reverse transcription system (Promega, Madison, WI). Briefly, 2  $\mu$ g of total RNA were transcribed to cDNA, adding 25 U of reverse transcriptase, 1  $\mu$ g of oligo dT, 5  $\mu$ l of 10 mM dNTP mixture, 20 U of RNasin, and 10  $\mu$ l of 25 mM MgCl<sub>2</sub>. The whole mixture was incubated at 42°C for 30 min.

The cDNA specific for inducible NOS (iNOS) was amplified by the PCR technique, using the Taq polymerase kit (Perkin-Elmer Cetus, Norwalk, CT) with the following primers specific for iNOS (up: 5'-CgTggAggCTgCCCggCagACTgg-3', down 5'-TgCCCg-gAAGgTTTgTACAgCCCCA-3') (22). Five microliters of cDNA were added to primers, MgCl<sub>2</sub>, dNTP, 1.2 U of Taq polymerase, and amplification buffer. Thirty cycles of amplification were performed (1 min at 60°C, 1 min at 94°C, 2 min at 60°C, and 3 min at 72°C). The cDNA was then run on a 1% agarose gel, and bands were visualized after ethidium bromide staining on an ultraviolet transilluminator. The specificity of the bands was confirmed by Southern blot, using iNOS cDNA. As a housekeeping control gene, we amplified mouse  $\beta$ -actin mRNA, using commercial primers (Clontech Laboratories, Palo Alto, CA).

### Cell Growth

[<sup>3</sup>H]thymidine incorporation assay was performed in 96-well microtiter plates in 200  $\mu$ l total volume. At the end of the 24-h incubation period with supplemented or unsupplemented media, cells were pulsed overnight with [<sup>3</sup>H]thymidine (1  $\mu$ Ci/well, 5 to 7 Ci/mmol; Amersham, Arlington Heights, IL). Cells were collected on glass microfiber filters (934-AH, Whatman, Clifton, NJ), using a cell harvester, and radioactivity was assessed after placing the filters in liquid scintillation fluid and counted in a  $\beta$ -counter.

### TNF Measurement

TNF- $\alpha$  was measured in supernatants of EC incubated for 6 h in various conditions by a commercial competitive immunoassay (T Cell Diagnostics, Inc., Cambridge, MA), according to the manufacturer's instructions. Our intra- and interassay coefficients of variation, evaluated in 10 different assays, were  $7 \pm 2$  and  $8 \pm 3\%$ , respectively. The levels of TNF- $\alpha$  were corrected for the protein cellular content and expressed as fold increases compared with values in unsupplemented media.

### Endotoxin Concentration

The concentration of endotoxin was determined in dialysate fluids at 405 nm by using Limulus Amebocyte Lysate (LAL) as a chromogenic substrate. The LAL was supplied by M.A. Bioproducts (Gaithersburg, MD), and the substrate was supplied by Kabi (Stockholm, Sweden); *Escherichia coli* O III: B4 was used as a standard.

The activity of 1 ng was equivalent to 12.5 endotoxin units (EU) of the U.S. reference standard EC-5. The sensitivity of the test was 0.01 ng/ml. Negative values were considered  $\leq 0.5$  EU/ml.

### Statistical Analyses

Values reported in the results represent mean  $\pm$  SD of five experiments, each performed in triplicate. Paired *t* test and Mann-Whitney nonparametric analysis were used to compare different conditions.

## Results

### NOS Activity in EC

Acetate at the standard concentration of the dialysis fluids (38 mmol/L) induced a  $3.2 \pm 0.39$ -fold increase of basal EC NOS activity ( $0.028 \pm 0.01$  pmol NO/min per mg protein) ( $P < 0.0005$ ) (Figure 1). Also, the small amount of acetate present in bicarbonate buffer (4 mmol/L) stimulated the basal EC NOS activity by a  $2 \pm 0.49$ -fold increase over basal values ( $P < 0.005$ ). AFB did not modulate the EC NOS basal activity.

As expected, LPS induced a  $22 \pm 2.4$ -fold increase of EC NOS basal activity ( $P < 0.0001$ ), and the addition of LPS to each dialysis buffer examined blunted the effect of the dialysis buffers on EC NOS activity.

### mRNA Encoding for iNOS Expressed by Cultured EC

Standard qualitative reverse transcription-PCR demonstrated a high expression of iNOS mRNA in EC incubated with acetate at the concentration of 38 mmol/L and even in the small amount present in the bicarbonate buffer (4 mmol/L), whereas AFB produced no effect on basal iNOS mRNA expression

(Figure 2). As expected, LPS was a potent enhancer of EC iNOS mRNA levels.

### TNF Synthesis of EC Incubated with Different Dialysis Buffers

Acetate induced a clear increase in TNF synthesis compared with the basal values ( $5 \pm 1.44$ -fold increase when cells were incubated with acetate,  $P < 0.0005$ ; and  $3 \pm 0.7$ -fold with bicarbonate,  $P < 0.005$ ) (Figure 3). AFB did not modify the basal EC TNF production. LPS was a powerful inducer of EC TNF synthesis with a  $20 \pm 2.34$ -fold increase of basal values ( $P < 0.0001$ ).

### EC Proliferative Index

Acetate at concentrations of both 38 and 4 mmol/L induced a significant reduction of the EC proliferative index ( $25 \pm 12$  and  $44 \pm 3\%$  of basal values;  $P < 0.0005$  and  $P < 0.005$ , respectively) (Figure 4). The EC proliferative index was not influenced by the incubation with AFB.

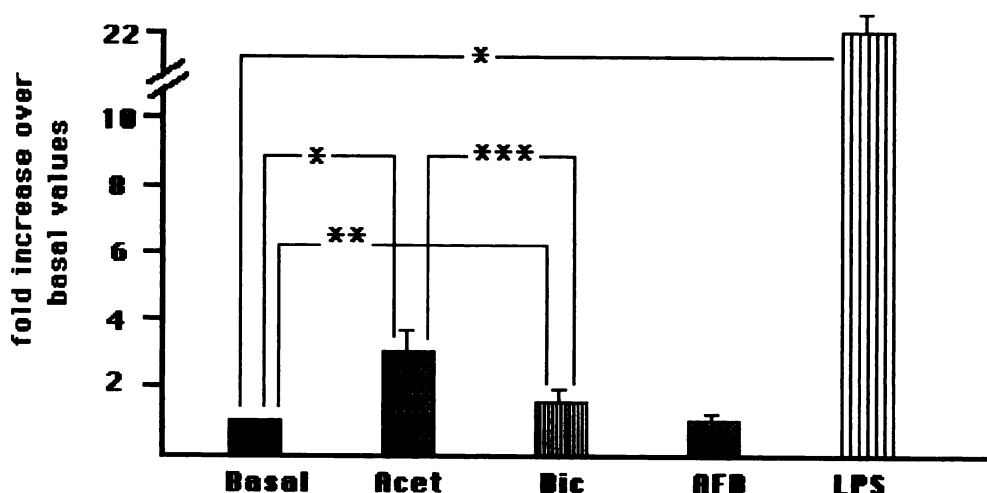
### Endotoxin Concentration

LAL was always negative in cell cultures incubated with the different dialysis buffers.

## Discussion

These data suggest a previously unconsidered mechanism for acetate to induce vasodilation during dialysis. We demonstrated that the coincubation of EC with acetate-buffered dialysis fluid at a concentration of 38 mmol/L, as well as with the small amounts present in bicarbonate buffer (4 mmol/L), significantly increased *in vitro* NOS activity. In contrast, the coincubation with AFB failed to modify NOS activity.

Mammalian cells have at least three genes encoding for each distinct isoform of NOS, which share 50 to 60% homology



**Figure 1.** Nitric oxide synthase (NOS) activity of endothelial cells (EC) after incubation with different dialysis buffers. Results are expressed as fold increase over basal values (mean  $\pm$  SD of five experiments). *P* values refer to nonparametric Mann-Whitney *U* test. Basal, NOS activity of EC in arbitrary units; Acet, acetate dialysis buffer (38 mmol/L); Bic, bicarbonate dialysis buffer (bicarbonate 35 mmol/L, acetate 4 mmol/L); AFB, acetate-free dialysis buffer; LPS, lipopolysaccharide (10  $\mu$ g/ml). \* $P < 0.0005$ ; \*\* $P < 0.05$ ; \*\*\* $P < 0.005$ .

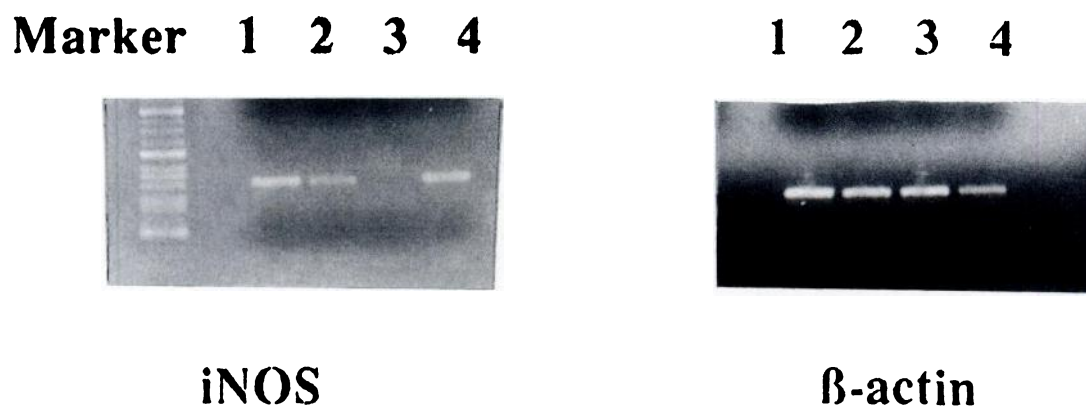


Figure 2. PCR amplification of specific mRNA encoding for inducible NOS (iNOS) and  $\beta$ -actin in EC after incubation with different dialysis buffers (representative of three separate experiments). Lane 1, acetate; lane 2, bicarbonate; lane 3, AFB; lane 4, LPS.

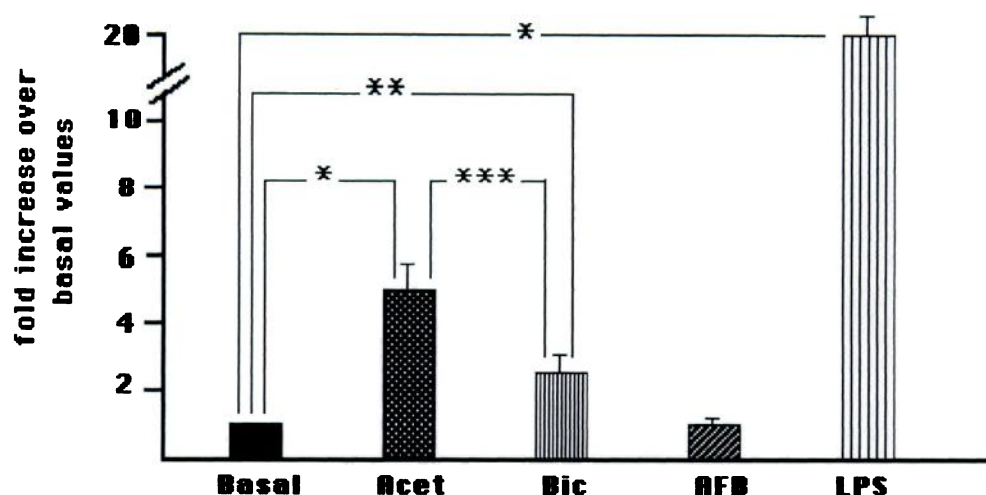


Figure 3. TNF- $\alpha$  release after incubation with different dialysis buffers. Results are expressed as fold increase over basal values. Abbreviations as in Figure 1. \* $P < 0.0005$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.05$ .

with one another at nucleotide and amino acid levels (23). Two NOS gene products are constitutively expressed: one in EC (eNOS) and a second in neuronal (nNOS) and other cell types, including kidney macula densa cells, skeletal muscles,  $\beta$  pancreatic cells, and epithelial cells of the lung. The third species of NOS is inducible by different stimuli in virtually all nucleated mammalian cells examined (iNOS). Although all NOS isoforms require bound calmodulin for activity, only the iNOS has a sufficiently high avidity for calmodulin to remain bound at the low calcium levels found in resting cells, conferring the full catalytic activity to iNOS (24). As a result, eNOS and nNOS probably produce small, physiological "puffs" of NO in response to transient elevation in intracellular calcium, whereas iNOS produces a large and continuous flux of NO until the substrate becomes limiting. On the basis of these observations, calcium-independent iNOS, rather than calcium-dependent constitutive eNOS and nNOS, is thought to be the isoform producing the large quantities of NO leading to tissue damage or death. Only in some pathologic conditions such as stroke (25) or after ischemia/reperfusion (26), a sufficiently continuous elevation in intracellular calcium may cause the

constitutive NOS isoforms to produce cytotoxic quantities of NO.

Because the measurement of NOS enzymatic activity does not distinguish constitutive NOS and iNOS, we focused our attention on iNOS, which, for the above considerations, could largely be involved in acetate intolerance. By using reverse transcription-PCR, we demonstrated high levels of the specific mRNA encoding for iNOS only in EC incubated with acetate or bicarbonate buffer containing trace amounts of acetate. Acetate increases production and release of IL-1 $\beta$ , and this effect is further enhanced when the cells are incubated with endotoxin (11). It is well known that IL-1 $\beta$  secretion can be induced by endotoxin or complement activation products (27) and that, in turn, IL-1 $\beta$  promotes TNF- $\alpha$  release (28) and many other immune system activities. These cytokines are thought to play a central role in various dialysis intolerance reactions, particularly in hemodialysis-associated hypotension (29,30). Recent evidence has been provided that TNF- $\alpha$  does not increase the constitutive NOS, but is a potent enhancer of the inducible form of the enzyme in various cellular systems (31,32).

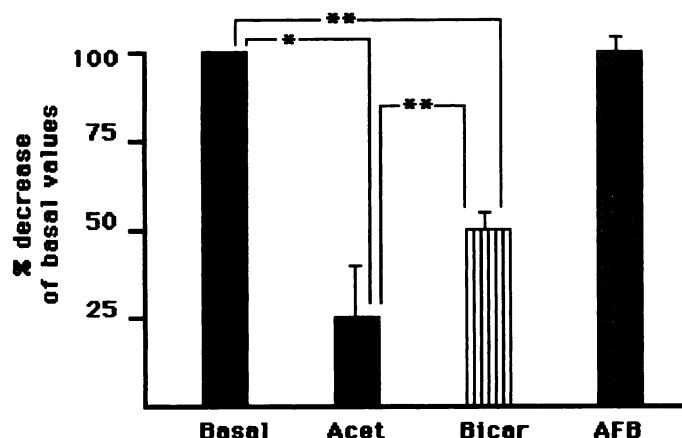


Figure 4. Proliferative index of EC grown after incubation with different dialysis buffers. Results are expressed as percent increase versus the basal values. Abbreviations as in Figure 1. \* $P < 0.0005$ ; \*\* $P < 0.005$ .

We demonstrated that acetate significantly increased endothelial TNF, whereas AFB was ineffective. The negative LAL test ensured the purity of the dialysis fluid used, thus excluding NOS activation by LPS. It is conceivable that the increase in iNOS mRNA levels and NOS enzymatic activity are a result of the induction of TNF by acetate in synergism with cAMP.

Because NO is an antiproliferative mediator (13), we evaluated the effect of acetate on the proliferative index of EC. Acetate buffer and bicarbonate buffer containing low amounts of acetate significantly reduced EC proliferation activity, whereas acetate-free solution failed to show any effect.

In conclusion, the data obtained support our hypothesis that acetate produces its vasodilatory effects by means of NO. The increase in cAMP and the stimulation of TNF- $\alpha$  transcription and translation suggest the possibility of a combined activation of constitutive NOS and iNOS.

Because the constitutive isoform of NOS functions in basal conditions, the effect of its stimulation is immediate and leads to rapid synthesis of NO. Therefore, the stimulation of constitutive NOS by acetate suggests the possibility of involvement of NO in intradialytic hypotension. Moreover, because we found an increase in iNOS mRNA levels, which may be due either to enhanced gene transcription or increased RNA stability, we propose that patients undergoing acetate dialysis, and even more when using poorly biocompatible membranes (33), are exposed to periodic stimulation of endothelial iNOS. Its final product, NO, could play a role in the development of long-term chronic vasculopathy of dialyzed patients.

Even though the clinical relevance of these “*in vitro*” studies has to be proved *in vivo*, it is worth noting that acetate, even in low concentrations, in bicarbonate buffer can modulate iNOS mRNA levels and enzymatic activity, whereas only dialysis solutions free of acetate do not activate this powerful mediator.

The small amounts of acetate present in bicarbonate dialysate, although capable of inducing *in vitro* NOS activation, are likely to be rapidly metabolized so that acetatemia does not stimulate NOS activity. Conversely, high acetate levels during

acetate dialysis overwhelm the hepatic metabolic capacity and are likely to lead to increased NO production with related clinical signs and symptoms.

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