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BH_4 (Tetrahydrobiopterin)-Dependent Activation, but not the Expression, of Inducible NOS (Nitric Oxide Synthase)-2 in Proinflammatory Cytokine-Stimulated, Cultured Normal Human Astrocytes Is Mediated by MEK–ERK Kinases

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Abstract Nitric oxide (NO) from astrocytes is one of the signalers used by the brain's extensive glial-neuronal-vascular network, but its excessive production by pro-inflammatory cytokine-stimulated glial cells can be cytodestructive. Here, we show how three pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) together stimulated the activation, but not the prior expression, of NOS-2 protein via a mechanism involving MEK–ERKs protein kinases in astrocytes from adult human cerebral temporal cortex. The cytokines triggered a transient burst of p38 MAPK activity and the production of NOS-2 mRNA which were followed by bursts of MEK–ERK activities, synthesis of the NOS-2 co-factor tetrahydrobiopterin (BH_4), a build-up of NOS-2 protein and from it active NOS-2 enzyme. Selectively inhibiting MEK1/MEK2, but not the earlier burst of p38 MAPK activity, with a brief exposure to U0126 between 24 and 24.5 h after adding the cytokine triad affected neither NOS-2 expression nor NOS-2 protein accumulation but stopped BH_4 synthesis and the assembly of the NOS-2 protein into active NOS-2 enzyme. The complete blockage of active NOS-2 production by the brief exposure to U0126 was bypassed by simply adding BH_4 to the culture medium. Therefore, this cytokine triad triggered two completely separable, tandem operating mechanisms in normal human astrocytes, the first being NOS-2 gene expression and accumulation of NOS-2 protein and the second being the synthesis of the BH_4 factor needed to dimerize the NOS-2 protein into active, NO-making NOS-2 enzyme. J. Cell. Biochem. 94: 731–743, 2005.

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Astrocytes make up about half of the volume of the central nervous system where they are parts of an extensive cellular network that modulates neuronal transsynaptic signaling and bidirectionally relays signals from neurons to vascular endothelial and muscle cells to adjust the blood flow and the passage of selected components across the blood–brain barrier in response to local bursts of neuronal activity [Ransom and Sontheimer, 1992; Braet et al., 2004]. Nitric oxide (NO) is one of the signalers in this extensive signaling network. As part of their normal duties, astrocytes make NO, which triggers Ca^{2+} waves and is thus involved in the propagation of calcium signals between astrocytes, other glial cells, neurons and beyond [Braet et al., 2004]. But when the brain is injured either by sudden stroke, the more slowly operating Alzheimer or Parkinson disease

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mechanisms or other processes, astrocytes respond to proinflammatory cytokines such as IL-1β, TNF-α, and IFN-γ from microglia by making more NO in an effort to scavenge reactive oxygen species (ROS) produced by the cytokine signaling and thus reduce damage to themselves, but they may release enough NO to kill their closely associated neuron neighbors [Beach et al., 1989; Norenberg, 1994; Dawson and Dawson, 1996; Lincoln et al., 1997; Grzybicki et al., 1998; Estévez and Jordan, 2002; Khaldi et al., 2002; Leong et al., 2002; Liu et al., 2002; Hirsch et al., 2003].

NO is a highly reactive free radical gas that is normally a signal messenger and can be a protective radical scavenger in an injured cell, but it can become destructive when generated excessively and prolongedly [Estévez and Jordan, 2002; Leong et al., 2002]. NO is produced from L-arginine by three types of NO synthase (NOS)—neuronal NOS-1 (or nNOS), inducible NOS-2 (or iNOS), endothelial cell NOS-3 (eNOS) [Lincoln et al., 1997]. Unlike the other two enzymes, NOS-2 is not made by resting cells such as astrocytes, but it is induced when the cells are activated by appropriate stimuli. Moreover, unlike the other two enzymes, it is unaffected by Ca^{2+} or $Ca^{2+} \cdot$ calmodulin although it does bind calmodulin [Lincoln et al., 1997]. NOS-2 is induced in astrocytes and other cells by proinflammatory cytokines [Suzuki et al., 1995; Grzybicki et al., 1998; Wada et al., 1998; Zamora et al., 2000], and NOS-2 is a major participant in the damaging of variously traumatized brains which can be reduced or prevented by blocking the enzyme's activation [Wada et al., 1998; Piao et al., 2003]. Therefore, to find drugs to limit stroke-reperfusion ROS injury for example, it would be useful to know how proinflammatory cytokines stimulate NOS-2 in astrocytes and how to prevent them from doing so.

Among the many NOS-2 inducers are the mitogen-activated protein kinases (MAPKs)—p38 MAPK and ERK1/ERK2 (p42/44 MAPK) [Johnson and Lapadat, 2002]. Piao et al. [2003] have found that selectively inhibiting p38 MAPK with SB203580 reduces cerebral infarct volume by as much as 50% in rats subjected to middle cerebral artery occlusion, and Bhat et al. [1998] have reported that both p38 and ERK1/ERK2 work together to induce active NOS-2 in the mixed cell populations of lipopolysaccharide-stimulated primary rat glial cell cultures. But it appears that it is p38 MAPK, rather than ERK1/ERK2, that is required for NOS-2 induction in mouse astrocytes and rat C-6 glioma cells stimulated by a combination of IFN-γ and TNF-α [Da Silva et al., 1997; Xu and Malave, 2000].

More recently Neufeld and Liu [2003] have found that the stimulation of NOS-2 gene expression in IFN-γ + IL-1β-treated adult human astrocytes also requires p38 MAPK but not ERK1/ERK2 activity because the expression was unaffected by the MEK–ERK kinase inhibitor PD98059. But was the NOS-2 produced in the cytokine-stimulated astrocytes treated with the MEK–ERK kinase inhibitor active? We now answer this question by showing that, as expected from Neufeld and Liu's results, inhibiting MEK–ERK kinase activity (with only a brief exposure to a selective MEK1/MEK2 inhibitor, U0126) does not prevent a trio of proinflammatory cytokines (i.e., IL-1β + TNF-α + IFN-γ) from inducing NOS-2 in the cells of pure cultures of normal adult astrocytes, but it does prevent the emerging NOS-2 proteins from being activated by preventing the cells from making the NOS-2-activator—tetrahydrobiopterin (BH_4).

MATERIALS AND METHODS

Isolation and Culturing of Phenotypically Normal Human Astrocytes

Small samples were taken from the edges of pieces of the normal parts of temporal lobe cortices from four patients three of who had perforating head injuries and one of them had a glioma. The samples were immersed in MCDB 153 medium (Sigma-Aldrich, Milan, Italy), put into a Dewar flask at 4°C and carried to the culture laboratory. There they were cut into tiny pieces, the cells in which were released by mild treatment with 0.25% (w/v) trypsin (Eurobio, Les Ulis Cedex, France) in Hank's Basal Salt Solution (BSS; Eurobio, France) at 18°C and triturated with a series of Pasteur pipettes with diameters decreasing from 5 to 1 mm.

The isolated cells were planted in culture flasks (BD Biosciences, Le Pont de Claix, France) containing a medium consisting of 89% (v/v) of a 1:1 mixture of Ham's F-12 and MCDB 153 media (Sigma-Aldrich), 10% (v/v) heat-inactivated (at 56°C for 30 min) fetal bovine serum (FBS; BioWhittaker Europe, Belgium) and 1% (v/v) of a penicillin–strepto-

mycin solution (Eurobio, France). Basic fibroblast growth factor (bFGF or FGF-2; 20 ng/ml; PeproTech EC Ltd., England), insulin-like growth factor-I (IGF-I; 20 ng/ml; PeproTech), platelet-derived growth factor (PDGF; 20 ng/ml; PeproTech), and epidermal growth factor (EGF; 10 nM; Sigma-Aldrich) were added to the medium to enhance the initial proliferation and selection of the astrocytes in the mixed cell population. This complete medium was replaced every 2–3 days.

When the primary mixed cultures became 70% confluent (1–4 weeks) the cells were detached from the flask surfaces with 0.25% (w/v) trypsin and 0.02% (w/v) EDTA (Eurobio) in Hank's BSS, split 1:4 and planted in new flasks. After the third subculture a homogeneous population of astrocytes appeared and the five growth factors were no longer needed. The cells of these pure cultures only expressed astrocyte-specific markers such as glial fibrillary acid protein (GFAP) and glutamine synthase (GS). None of the cells expressed neuronal (enolase), oligodendrocytes (galactocerebroside), microglia (CD-68), or endothelial cells (factor VIII) markers. These astrocytes proliferated slowly without added growth factors in serum-enriched Ham's F-12/MCDB 153 medium. Thus, withdrawing growth factors after the third passage did not prevent the astrocytes from proliferating to confluence and expressing their characteristic astrocyte markers—they were by now phenotypically "locked in." But serum remained necessary and withdrawing it caused the astrocytes to self-destruct by apoptosis. The proliferatively quiescent cells in confluent astrocyte cultures started cycling again when subcultured. At least 15–18 subcultures could be obtained over 2.5 years from a piece of normal cortex. Only astrocytes from the fourth to the eighth subculture were used because the response of the cells to proinflammatory cytokines became erratic with further subculturing.

Experimental Protocol

Since astrocytes are normally not proliferating in the adult human brain when they are assailed by injury-induced proinflammatory cytokines, we studied the NOS-2 induction by such cytokines using confluent, proliferatively quiescent pure astrocyte cultures. Thus, at "time-0," some such cultures served as untreated controls while others had IL-1β (20 ng/ml) alone and/or TNF-α (20 ng/ml) plus IFN-γ (70 ng/ml) (all from PeproTech) added to their medium. In some cases TNF-α and/or IFN-γ were added without IL-1β. In some experiments a second, third, or fourth bolus of the three cytokines was added to the cultures' media at 24, 48, or 72 h.

In the first set of experiments U0126 (1, 4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; 10 μM; Promega, Italy), a selectively targeting, long-acting, non-competitive inhibitor of the dual specificity MEK1/MEK2 (and more recently MEK5) protein kinases and their phosphorylated, i.e., active forms [Alessi et al., 1995; Favata et al., 1998; Kamakura et al., 1999; Davies et al., 2000] were added for 30 min and then washed out just before the cytokines were added and then added again at 24 h and washed out at 24.5 h. To do this the cell-conditioned cytokine-free medium was carefully removed and medium containing 10 μM U0126 was put on the cultures for 30 min after which it was removed by washout and the original cell-conditioned U0126-free, but now cytokine-containing medium was put back on the cultures and the experiment began. This was repeated, washout included, for another 30 min at 24 h after adding the cytokines. This was done to block any MEK–ERK response to the signals from cytokine receptors during the first 24 h of the cells' response to the cytokines. The brief U0126 exposure protocol was also meant to avail ourselves of the inhibitor's selective targeting of MEK kinases while avoiding any side-effects of prolonged exposure to the inhibitor. However, to find out when during the first 24 h the MEK–ERKs entered into the NOS-2/NO responses to the cytokines U0126 was added only once in later experiments, either for 30 min and then washed out just before adding the cytokines or at 24 h after adding the cytokines and washed out 30 min later.

Measurement of Intracellular NO Production

Intracellular NO production was determined using DAF-FM diacetate (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; Molecular Probes Europe, The Netherlands) [Kojima et al., 1998]. This cell-permeating compound is non-fluorescent, but when it enters the cell the acetates are removed by the cells' esterases and it reacts with NO form a fluorescent benzotriazole [Kojima et al., 1998; Itoh et al., 2000]. A DMSO stock solution of DAF-FM diacetate was

diluted with a solution consisting of 99% (v/v) PBS and 1% (v/v) FBS to contain 10 μM DAF-FM diacetate. Untreated and cytokines-treated astrocytes were incubated in this diluted DAF-FM solution in tissue culture chamber slides (Nunc) for 60 min at 37°C and then photographed using a fluorescence microscope (excitation and emission maxima 495 and 515 nm, respectively). Deconvoluted fluorescence images were obtained by using Huygens Essential software for Windows (Scientific Volume Imaging b.v., Hilversun, The Netherlands). The same cells were also photographed under phase contrast optics to measure the fraction of cells making NO.

Measurement of NO Released Into the Medium

The amount of NO that the cells dumped into their medium was determined from the concentrations of NO's two stable oxidation products, NO_2^- and NO_3^- in the medium. The fluorometric method we used is based upon the reaction of NO_2^- with 2,3-diaminonaphthalene (DAN; Sigma-Aldrich) to form the fluorescent 1-(H)-naphthotriazole [Misko et al., 1993]. Nitrates in the medium were reduced to NO_2^- by incubation for 30 min with nitrate reductase (0.1 U/ml; Boehringer Mannheim) in the presence of 10 μM FAD and 100 μM NADPH. Any residual NADPH was then oxidized with lactate dehydrogenase (10 U/ml) in the presence of 10 mM sodium pyruvate. The total NO_2^- concentration was then determined in 50 μl samples of the culture supernatants that were brought up to 100 μl with doubly deionized water. Freshly prepared DAN (10 μl of a 0.05 mg/ml solution in 0.62M HCl) was added to these diluted samples and, after a 10 min incubation at 20°C in the dark the reaction was stopped with 5 μl of 2.8N NaOH. The amount of 1-(H)-naphthotriazole formed was measured fluorometrically at excitation and emission wavelengths of 365 and 450 nm, respectively. Samples of fresh culture medium were used as blanks to correct for background NO_2^- and NO_3^- concentrations in the medium (~7 μM). NO_2^- concentrations were calculated from a standard curve using $NaNO_2$.

mRNA Isolation and Northern Blot (NB) Analysis of NOS Isoform mRNAs

After 0–48 h incubation with or without cytokines (i.e., 20 ng/ml IL-1β plus 20 ng/ml TNFα and 70 ng/ml IFN-γ, astrocytes were lyzed with SV RNA lysis buffer reagent (Promega Italia, Milan), and their total RNA isolated according to the manufacturer's instructions for the SV Total RNA Isolation System (Promega). RNA concentration was measured with a Uvikon 922 spectrophotometer equipped with a 5-carat microcell. For NB analysis, samples of total RNA (20 μg/lane) were mixed with ethidium bromide (1–2 μg) and loaded onto a 1.2% denaturing agarose gel containing formaldehyde. After separation by electrophoresis, RNA was transferred to a Hybond-N^+ nylon membrane (Amersham Biosciences, Piscataway, NJ) and UV cross-linked for hybridization at 33°C with double-FITC-labeled probes (HybriProbes; Biognostik GmbH, Göttingen, Germany) specific for the NOS isoforms mRNAs. To significantly enhance the sensitivity of the assays for the very rare NOS-1 and NOS-3 mRNA transcripts, we used a combination of three different double-FITC-labeled HybriProbes, each with a different sequence for the corresponding target mRNA. On the other hand only one sequence was needed to probe for the far more plentiful NOS-2 mRNA transcripts.

The sequences of these HybriProbes were:

(i) for NOS-1: 5′-ACGCTGTTGAATCGGAC-CTTGTAGCTCTT-3′; 5′-TCCAGGTA GT-ACTTGAAGGCCTGGAAGATG-3′; and 5′-TTCCCTTTGTTGGTGGCATACT TG-ACATGG-3′;
(ii) for NOS-2: 5′-CCTGGCCAGATGTTCCTC-TATTTTTGCCTC-3′;
(iii) for NOS-3: 5′-ATGGAGCTGTAGTACT-GGTTGATGAAGTCC-3′; 5′-CTGCAGGA CGTTGGTTGCCATGGTAACAT-3′; and 5′-TGAGTCCTGTATGCCAGCACA GCT-ACAGT-3′.

The hybridization reactions were performed with the HybriBuffer N hybridization solution (Biognostik) according to the manufacturer's instructions. After hybridization, each filter was washed three times in 2× SSC (1× SSC: 150 mM NaCl, 15 mM Na_3-citrate · $2H_2O$, pH 7.0 with 1M HCl), 0.1% SDS for 30 min at 20°C and once in 0.1× SSC, 0.1% SDS solution for 15 min at 40°C. The contents of the hybridized membranes were then analyzed using the Southern Star™ (Tropix, Applied Biosystems, Framingham, MA) chemiluminescent detection system based upon an anti-FITC alkaline phosphatase

conjugate antibody and the CDP-STAR substrate (Tropix, Applied Biosystems) following the manufacturer's instructions. The developed membranes were photographed with an Olympus 3300^{TM} digital camera. The membranes were next stripped by boiling in 0.1× SSC, 0.1% SDS for 20 min and hybridization repeated using a probe for actin's mRNA (Biognostik) as an internal control for the amount of total RNA loaded onto each lane.

Western Immunoblotting (WB)

Twenty-four, 48, or 72 h after starting the experiments, control and treated astrocytes were scraped into cold PBS and sedimented at 200*g* for 10 min. The sedimented cells were homogenized in T-PER^{TM} tissue protein extraction reagent (Pierce Chemical Co., Rockford, IL) containing a complete EDTA-free protease inhibitor cocktail (Roche Diagnostics SpA, Monza, Italy). The protein contents were assayed with an ad hoc commercial kit (Pierce Chemical Co.). Equal amounts (25–30 μg) of protein from the samples were boiled in buffer (0.0625M Tris-HCl, pH 6.8; 2% (w/v) SDS; 5.0% (w/v) β-mercaptoethanol; 10% (v/v) glycerol; and 0.002% (w/v) bromphenol blue), electrophoresed in 10% (w/v) SDS–polyacrylamide gel, and blotted onto nitrocellulose membranes (0.45 μM; Pall Life Sciences, Ann Arbor, MI).

To immunodetect NOS-1, NOS-2, NOS-3, Raf1, phospho-Raf1, MEK1/MEK2, phospho-MEK1/MEK2, ERK1/ERK2, phos-pho-ERK1/ERK2, p38-MAPK, and phospho-p38 MAPK, the blots were probed with specific rabbit IgG polyclonal antibodies or mouse monoclonal antibodies at a final dilution of 1 μg/ml (Santa Cruz Biotechnology, Inc.). For NOS-2 protein detection, a positive control—a protein lysate from cytokines-activated human macrophages (also from Santa Cruz Biotechnology, Inc.)—was run in parallel with the samples. Blots were also probed with goat/rabbit polyclonal antibodies (final dilution 1 μg/ml; Santa Cruz Biotechnology, Inc.) to monitor the specific astrocyte markers GFAP and GS. The blots were next incubated with alkaline phosphatase-conjugated anti-goat or anti-rabbit IgGs (Santa Cruz Biotechnology, Inc.) and stained with BCTP/NBT liquid substrate reagent (Sigma). The developed blots were photographed, and the M_r and density of each band were measured using Sigmagel^{TM} software (Jandel Corp., Erkrath, Germany).

Immunocytochemistry (IC)

Immunostaining astrocytes that had been seeded into 24-well plates for primary tissue cultures (Becton Dickinson & Co., Franklin Lakes, NY) was carried out at 4°C. Astrocytes (2.0×10^4/chamber) were washed twice with PBS (phosphate-buffered saline) containing BSA (1.0% w/v) and NaN_3 (0.1% w/v), and incubated for 60 min at room temperature with primary antibodies (at 1.0 μg/ml) against NOS-2, active (i.e., phosphorylated) (phospho)-ERK-1/ERK-2 (all from Santa Cruz Biotechnology, CA). The cells were washed three times with PBS-BSA solution and then incubated for 60 min at room temperature with specific secondary antibodies conjugated to rhodamine or alkaline phosphatase (all from Santa Cruz Biotechnology). In the latter case, specific IC colors were developed with Fast Red AS-MX tablet sets (Sigma-Aldrich). After a final wash with PBS–BSA solution, specimens were examined under an inverted Zeiss IM35 microscope and photographed with an Olympus 3300^{TM} digital camera. Appropriate controls were always run in parallel without primary or secondary antibody. Deconvoluted fluorescence images were obtained as previously indicated (see "Measurement of Intracellular NO Production").

BH_4 Assay

BH_4 concentrations in growth medium and cell lysates were determined, after selective acid or alkaline oxidation with iodine according to Fukushima and Nixon [1980]. Aliquots (100 μl) of cell-conditioned medium or cell lysates were mixed with either 10 μl of acid iodine solution containing 0.5% iodine and 1% potassium iodide in 0.2M trichloroacetic acid (tube A) or with 2M NaOH and alkaline iodine solution (1% iodine and 2% potassium iodide in 0.1M NaOH) (tube B) and incubated for 1 h at room temperature in the dark. The oxidation reactions were terminated by adding 10 μl of a freshly made 1% solution of ascorbic acid, and the mixtures were centrifuged at 10,000*g* for 10 min. The supernatant was diluted (1:5) with distilled water and applied to a reverse-phase HPLC Luna C18 column (4.6 × 150 mm; Phenomenex Inc., CA), and eluted isocratically with 10 mM Na-acetate buffer (pH 5.2) containing 0.1 mM EDTA at a flow rate of 0.8 ml/min. The eluate was monitored with a fluorescence detector (excitation at 350 nm, emission at 440 nm). The BH_4

concentration in every sample was calculated by subtracting the amount of biopterins in the aliquot that had undergone alkaline oxidation (i.e., biopterin plus 7,8-dihydrobiopterin) from the amount of biopterins in the aliquot subjected to acidic oxidation (i.e., biopterin plus 7,8-dihydrobiopterin plus BH_4).

Statistical Analysis

A one-way analysis of variance (ANOVA) with post hoc Bonferroni test to compare mean values was applied to the data and $P < 0.05$ was considered to be significant.

RESULTS

Selective NOS-2 Induction by Proinflammatory Cytokines

Neither IL-1β nor TNF-α + IFN-γ induced the adult human astrocytes to make NO (Fig. 1A). But the CM trio of IL-1β + TNF-α + IFN-γ did stimulate the cells to make NO and release it into the medium (Fig. 1A). A single exposure to the CM cytokine trio was the most effective stimulator of NO release into the medium—two, three, or four additions of the triad to the culture medium at 24, 48, and/or 72 h did not further increase the amount of NO released into the medium by day 6 (Fig. 1B).

As expected from the untreated astrocytes' failure to make NO, they had no detectable NOS-mRNA species as assessed by NB analysis (Co gel lane; Fig. 2A), nor did they have any detectable NOS proteins as assessed by Western blot analysis (Co gel lane; Fig. 2B), and no NO-specific intracellular fluorescence appeared when these untreated cells were incubated with DAF-FM diacetate indicator (Co panel; Fig. 2C).

When the CM trio was added to the astrocyte cultures, NOS-2 mRNA appeared in the cells by 48 h (CM lane; Fig. 2A) and NOS-2 protein appeared by 72 h (CM band; Fig. 2B). NO appeared in the cytoplasms of about 85% of the cells along with NOS-2 protein between 48 and 72 h (CM lane; Fig. 2C) and they began releasing the gas into the medium in a sharp burst (about 0.7 μM/day) between days 2 and 3, but the rate of release then dropped to only about 0.03 μM /day between days 3 and 8 (Fig. 3A).

In contrast to NOS-2, the CM-treated astrocytes did not detectably make NOS-1 or NOS-3 mRNA even when the sensitivities of the NB analyses were tripled by using a mixture of three different probes for NOS-1 and NOS-3 mRNA transcripts, nor did the cells have any detectable NOS-1 and NOS-3 proteins even when the sensitivities of the Western blot

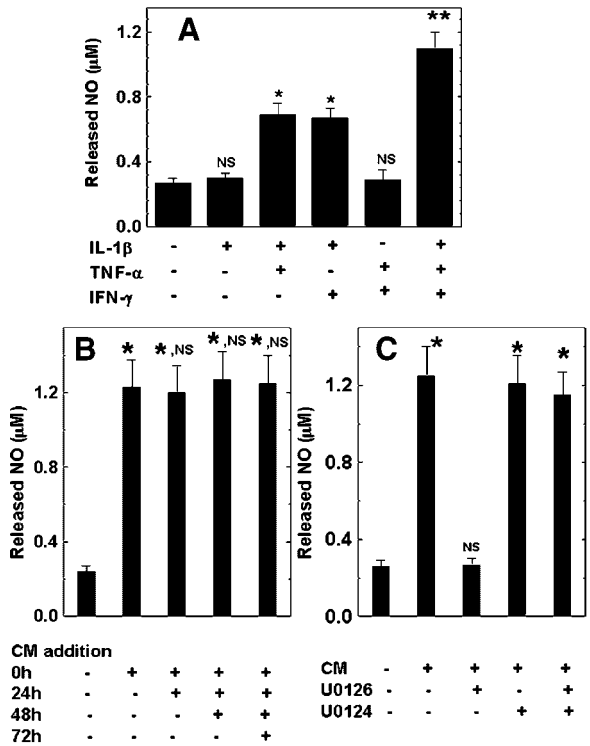

Fig. 1. Three proinflammatory cytokines, IL-1β (20 ng/ml), TNF-α (20 ng/ml), and IFN-γ (70 ng/ml), added together as the CM trio, maximally induced the generation of nitric oxide (NO) and its release into the medium of pure cultures of adult human astrocytes, a response that was totally prevented by two short exposures to the MEK–ERK inhibitor U0126, but not by exposures to U0126's inactive analog, U0124. **A**: Various combinations of the three cytokines were added to pure fourth to eighth passage astrocyte cultures at time"0." The concentrations of nitrate and nitrite derivatives of NO in medium samples were measured fluorometrically and then converted to NO equivalents as described in "Materials and Methods." Bars are the mean ± SEM of the values from at least three separate experiments. The significance levels for the various cytokine treatment data with respect to untreated controls: NS, not significant; $*P < 0.01$, $**P = 0.001$. **B**: Adding the CM trio again at 24, 48, and 72 h did not further increase the amount of NO released into the culture medium by day 6. The mean ± SEM NO levels in the CM trio-treated cultures were not significantly different from each other (NS) but were significantly different from the mean NO level in the untreated controls ($*P < 0.01$). **C**: Two short exposures to 10 μM U0126 between −0.5 and 1 h before adding the CM triad and again between 24 and 24.5 h after adding the trio followed by washouts prevented the triad from stimulating NO production. Two short exposures to U0126's inactive analog U0124 did not stop the trio from stimulating NO synthesis, but adding U0124 with U0126 prevented U0126 from inhibiting NO production. As in the other panels, the bars are the mean ± SEM of the values from at least three separate experiments. NS, not significant from the control values; $*P < 0.001$.

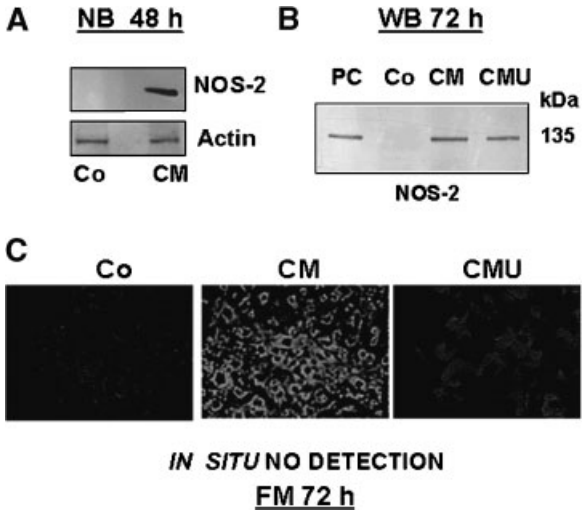

Fig. 2. Two 30-min exposures to and washouts of U0126 inhibited the activation, but not the induction, of NOS-2 in normal adult human astrocytes. At 0 h the CM trio of IL-1β (20 ng/ml), TNF-α (20 ng/ml), and IFN-γ (70 ng/ml) was added to fourth to eighth passage cultures. **A**: NOS-2 mRNA was detected with Northern blotting (NB) in the CM trio-treated astrocytes, but not in untreated controls (Co), at 48 h after adding the cytokines. Actin mRNA served as an internal control for the amount of RNA loaded onto the filter. **B**: Equal amounts of NOS-2 proteins were detected by Western blotting (WB) in the CM trio-treated (CM gel band) and U0126 + CM trio-treated (CMU gel band) cells, but not in untreated control (Co) cells, by 72 h after adding the cytokines. PC: Positive control, a NOS-2 standard consisting of a protein lysate from cytokines-activated human macrophages. **C**: Cytoplasmic NO in the cytokines-treated (CM), but not in the controls (Co), or the CM trio + U0126-treated (CMU) cells, could be seen from 72 h onward using the indicator DAF-FM and fluorescence microscopy (FM; original magnification, 100×). Control cells had only a weak autofluorescence. The deconvoluted images shown here were obtained as indicated in the ''Materials and Methods.'' It should be noted that if a significant amount of U0126 had remained in the cells after the second exposure between 24 and 24.5 h, the U0126-treated cells in the CMU panel would have been fluorescing brightly under these conditions [Blank et al., 2002].

analyses were increased 5-fold by loading five times more protein onto each lane.

Induction of BH_4 Synthesis

Since the production of active homodimeric NOS-2 molecules requires the BH_4 co-factor [Werner et al., 1993, 1998, 2003; Tzeng et al., 1995; Thony et al., 2000], we expected a surge of BH_4 synthesis to accompany the surge of NO production. But, since cells can dump BH_4 into their medium faster than they make it [Fukushima and Nixon, 1980; van Amsterdam et al., 1996; Choi et al., 2000], we measured its accumulation in both the culture medium and the cells.

Untreated astrocytes released very little BH_4 into the medium for the first 3 days (Fig. 3B). CM-treated cells also released very little BH_4 into the medium during the first day (Fig. 3B). But then between days 1 and 2 the CM-treated cells began releasing significantly more BH_4 and between days 2 and 3 the release rose dramatically along with NO production (Fig. 3A,B). By day 3 the concentration of BH_4 in the medium was 82.5 times the starting concentration (Fig. 3B). The buildup of BH_4 in the CM-treated cells paralleled the release of BH_4 into the medium but at lower levels (Fig. 3B, inset).

MAP Kinase Cascade: Raf–MEK–ERK

The total amounts of Raf1, MEK1/MEK2, and ERK1/ERK2 proteins did not change between 0 and 72 h after adding the CM triad to the astrocyte cultures, and as expected from basally functioning cells, the untreated astrocytes started out with low, but detectable levels of activated (i.e., phosphorylated) components of the RAF–MEK–ERK machinery (Figs. 4–6).

By 72 h the active phospho-MEK1/MEK2 fractions had equally more than more doubled ($P < 0.001$) (Fig. 4). The active phospho-ERK1/ERK2 fractions stayed at the control level during the first 24 h in the CM trio-treated astrocytes, but they began rising between 24 and 48 h (Fig. 5). The phospho-ERKs first appeared in "granules" around the periphery of the cytoplasm by 48 h, but by 72 h they had spread throughout the cytoplasm (Fig. 5C–E). According to Western blot densitometric analysis the phospho-ERK1/ERK2 fractions, but mostly the phospho-ERK2 fraction, had increased 7.5-fold ($P < 0.01$) by 72 h (Fig. 6A).

Perhaps surprisingly, the level of active phospho-Raf1, the canonical initiator of the MEK–ERK cascade [Pearson et al., 2001], had not increased by 72 h (Fig. 6B).

Impact of MEK-Inhibition by U0126 on NOS-2 Activation

We then addressed the question of what the late Raf1-independent burst of MEK–ERK activity had to do with NOS-2 induction and activation. To do this we selectively shut down the MEK1/MEK2 kinases and with them the ERK1/ERK2 kinases they activate, but not p38 MAPK, during the first 24 h of the response to CM addition by adding U0126 first from −30 min to 0 h and then washing it out and once

again from 24 to 24.5 h and then washing it out again (as described in "Materials and Methods").

The two brief exposures to U0126 did not change the total amounts of Raf1, MEK1/MEK2, and ERK1/ERK2 proteins in the CM-treated cells for 72 h after adding the CM triad (Fig. 4) but, the inhibitor caused phospho-MEK1/ERK2 to drop 80% by 24.5 h (the faint bands in the CMU gel of Fig. 4). However, as expected the phospho-MEKs had almost got back to the basal level by 72 h (i.e., 2 days after the second U0126 washout) (Fig. 4).

The brief exposures to U0126 did not affect the amount of phospho-Raf1 at 24 h (Fig. 6), but they completely prevented phospho-MEK1/MEK2 and phospho-ERK1/ERK2 from rising to high levels by 72 h as they did in CM trio-treated cells without the inhibitor (Figs. 4–6). However, despite having completely prevented the CM trio from stimulating the MEKs–ERKs, the two brief exposures did not stop the CM trio from stimulating the expression of NOS-2 (CMU lane; Fig. 2B). But this NOS-2 must have been inactive because the U0126 exposures also completely prevented NO production for as long as 6.5 days (Fig. 3). As required for a specific targeting action of U0126, two exposures to its inactive structural analog, U0124, did not stop the CM trio from stimulating NO production, but it prevented U0126 from inhibiting NO production (Fig. 1C).

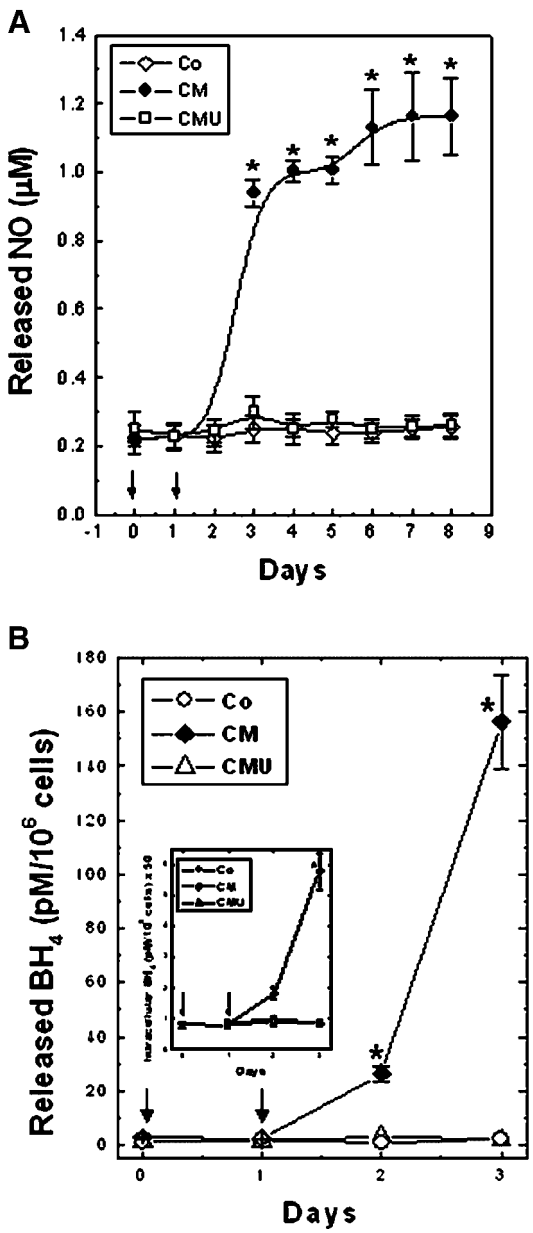

Fig. 3. The release of NO and BH_4 into the growth medium coincided in CM trio (IL-1β: 20 ng/ml, TNF-α: 20 ng/ml, and IFN-γ: 70 ng/ml)-adult human astrocyte cultures and both were prevented by two brief exposures to, and washouts of U0126 (arrows). **A**. The flow of NO into the cultures medium from the CM trio-treated astrocytes was suppressed completely during the first 6.5 days after the second of the two exposures to U0126. The concentrations of nitrate and nitrite derivatives of NO were measured to give the NO concentrations as described in "Materials and Methods." The points are mean ± SEM of the values from at least three separate experiments; $*P < 0.001$. **B**: Untreated control cells (Co) released little or no BH_4 into their medium for at least 3 days. But stimulating the cells with the CM trio (CM curve) caused them to make BH_4 and dramatically start dumping it into the medium along with NO between 1 and 2 days later. Two exposures to U0126 did not stop the expression of NOS-2 protein (Fig. 2) but it stopped the cells from making and releasing the NOS-activating BH_4 (CMU curve). Inset: The intracellular levels of BH_4 mirrored, but at much lower levels than the amounts of the co-factor dumped into the medium and the inhibition of its synthesis by the two exposures to U0126. The BH_4 concentrations in the cell extracts and culture medium were measured by reverse phase HPLC after selective acid or alkaline oxidation with iodine as described in "Materials and Methods." The points are the mean ± SEM of the values from three separate experiments, each one in duplicate; $*P < 0.001$.

When did the CM Trio-Stimulated Astrocytes Need MEK–ERK to Make NO?

Briefly exposing CM-treated astrocytes to U0126 only once, between −30 min and 0 h, before adding the CM trio did not prevent them from releasing a normal amount of NO into the medium (Fig. 7). But exposing them to U0126 only once between 24 and 24.5 h after CM addition when phospho-MEK1/MEK2 and phosphoERK1/ERK2 were still at their basal levels (Figs. 4 and 5), prevented the stimulation of NO production as effectively as the two brief exposures to the inhibitor (Fig. 7). Thus, the CM

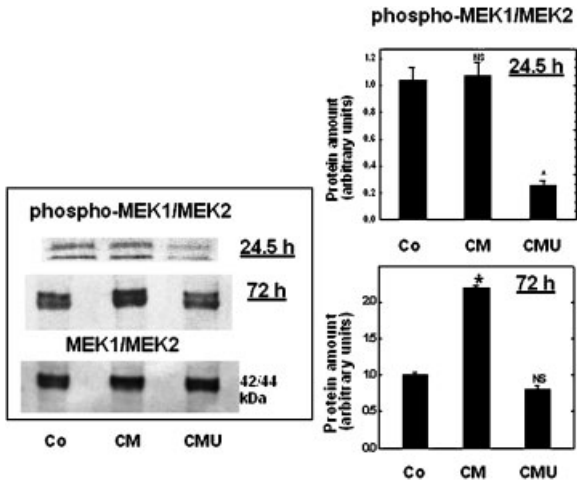

Fig. 4. The effect of two brief exposures to, and washouts of U0126 on MEK1/MEK2 activation (i.e., phosphorylation). MEK1/MEK2 had not yet responded to the CM trio by 24 h as shown by the upper row of gel immunoblot bands in the **left panel** and the band densities in the **upper right panel**. While the total MEK levels in the CM-treated cells stayed unaffected by 72 h (the lower immunoblots in the left panel and the **lower right panel**), the level of phosph-MEK1/MEK2 had risen to twice the control level by 72 h after adding the trio. While the total MEK levels were also unaffected by U0126, the level of phospho-MEK1/MEK2 by the time of the second and last U0126 exposure and washout at 24.5 h was only about 20% of the control level, but it had almost returned to the control level by 72 h. Left panels are typical Western immunoblots from three separate experiments and the panels on the right are the relative densities of the immunoblot bands determined as described in ''Materials and Methods.'' The heights of the relative density bars in the right panels are the mean ± SEM of three separate experiments. Abbreviations: Co, control; CM, CM trio-treated; CMU, CM, trio- and U0126-treated. *$P < 0.001$.

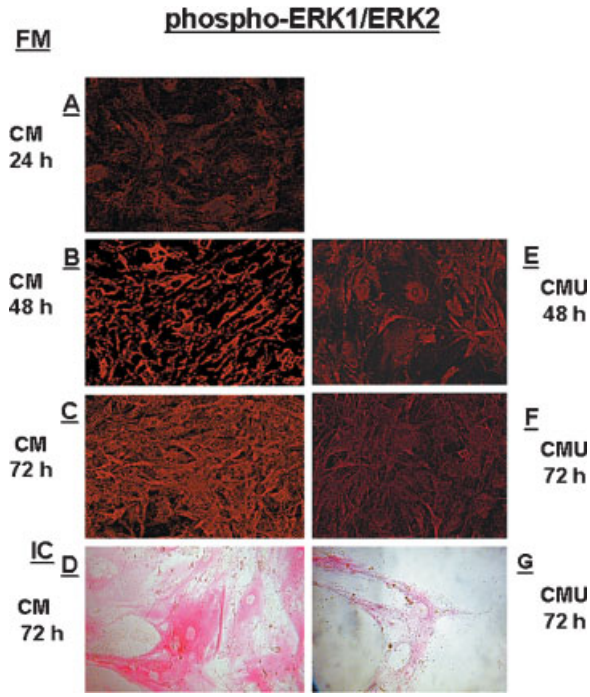

Fig. 5. The activation of ERK1/ERK2 following the addition of the CM trio (IL-1β: 20 ng/ml, TNF-α: 20 ng/ml, and IFN-γ: 70 ng/ml). The phospho-ERKs began appearing on the cell peripheries around 48 h after the CM trio was added and then spread throughout the cytoplasms by 72 h. Two brief exposures to, and washouts of U0126 prevented the ERK activation. The inhibition of ERK activation by U0126 is strikingly illustrated by comparing the phospho-ERKs-loaded cytoplasms of the CM-trio treated cells in **panels B–D** with the nearly phospho-ERKs-less cytoplasms of the U0126-/CM-trio-treated cells in **panels E–G**. The deconvoluted fluorescent images shown here are typical of at least three distinct experiments and were obtained as indicated in the ''Materials and Methods.'' Original magnifications: A–F, 100×; G, 200×.

trio-treated astrocytes needed a U0126-sensitive process between 24 and 24.5 h to make NO.

MEK–ERK Signaling and BH_4 Induction

The fact that NO production, but not the production of NOS-2 protein, in the CM trio-treated astrocytes required U0126-sensitive MEK–ERK activity raised the possibility of the failure to activate the enzyme being due to a lack of BH_4, the co-factor that drives the homodimerization of pre-enzyme NOS-2 proteins into NO-making NOS-2 dimers. Indeed U0126 did prevent the CM trio-treated cells from making both BH_4 (Fig. 3B) and NO (Figs. 3A, 7, and 8). But adding 100 μM BH_4 to astrocyte cultures along with the CM trio completely prevented U0126 from blocking NO production, as indicated by the nearly normal amount of NO released from U0126-treated cells into the BH_4-supplemented medium by 5 days after adding the CM trio (Fig. 8). It should noted that the control cells in otherwise untreated control cultures were unaffected by 100 μM BH_4.

CM-Induced Early Burst of p38 MAPK Activity

According to the results of these experiments there was a late, U0126-inhibitable, burst of MEK–ERK activities that did not affect the CM trio-triggered expression of NOS-2, but was needed for the synthesis of BH_4 to activate the emerging NOS-2. Thus to reach the U0126-sensitve stage the CM trio must have triggered an earlier, U0126-insensitive mechanism that stimulates the expression, but not the activation, of NOS-2. Since it is known that the U0126-insensitive p38 MAPK is involved in stimulating NOS-2 gene expression in normal

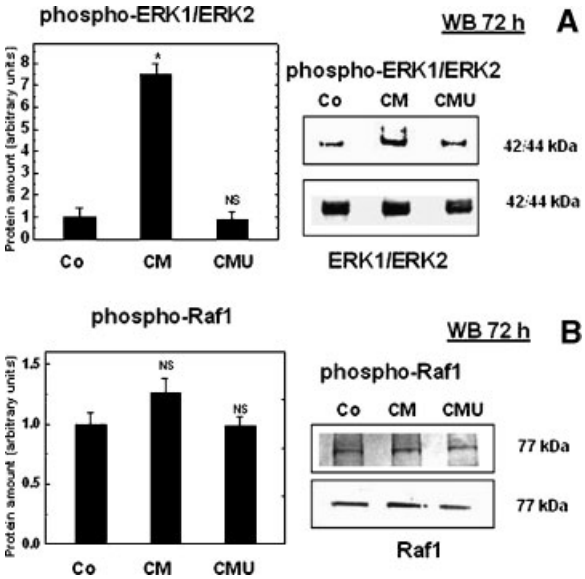

Fig. 6. The Raf1-independent ERK activation by the CM trio and its suppression by two brief exposures to, and washouts of U0126. **A**: The striking 7.5-fold increase in active phospho-ERK2 by 72 h after adding the CM trio (IL-1β: 20 ng/ml, TNF-α: 20 ng/ml, and IFN-γ: 70 ng/ml) as seen with Western immunoblotting (WB) was prevented by the two brief exposures to U0126 at −0.5 to 0 h and 24–24.5 h. **B**: Treating the cells with the CM trio or the CM trio and U0126 did not affect Raf1. The immunoblots were produced and the gel band densities obtained as described in ''Materials and Methods.'' The immunoblots are typical of the ones from three separate experiments. The heights of the bars are the mean ± SEM of the values from three separate experiments. $^*P < 0.001$. Abbreviations: Co, control; CM, CM trio-treated; CMU, CM, trio- and U0126-treated.

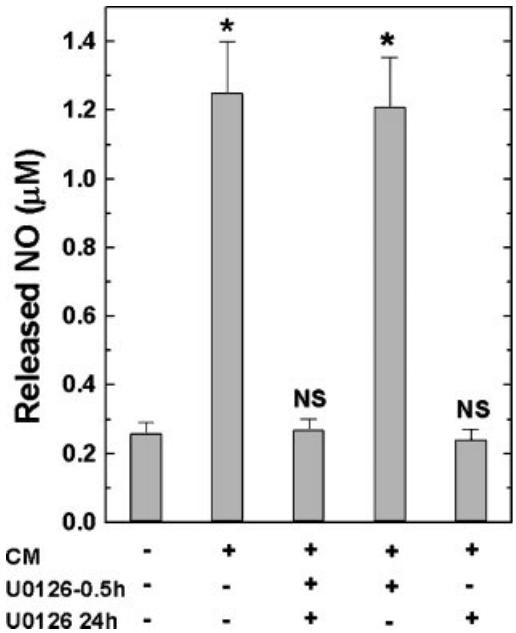

Fig. 7. When the CM trio-stimulated needed MEK–ERK activity to make NO. Adding 100 μM U0126 briefly between −0.5 and 0 h before adding the CM trio (IL-1β: 20 ng/ml, TNF-α: 20 ng/ml, and IFN-γ: 70 ng/ml) and then washing the inhibitor out did not affect the amount of NO the astrocytes released into the culture medium by 8 days later. However, briefly adding the inhibitor to the culture medium between 24 and 24.5 h after adding the CM trio completely prevented the stimulation of NO synthesis. In fact, the single 24–24.5 h exposure to U0126 was no less inhibitory than the two exposures at −0.5 and 0 h and 24–24.5 h. The concentrations of nitrate and nitrite derivatives of NO in the medium were measured to give the NO concentration as described in ''Materials and Methods.'' The bars are the values from at least three separate experiments. NPS, not significantly different from the values in the media of control CM trio-treated cultures; $^*P < 0.001$.

human astrocytes stimulated by IL-1β + IFN-γ [Neufeld and Liu, 2003], we measured active phospho-p38 MAPK in our astrocytes after adding the CM triad.

The total amount of cellular p38 MAPK did not change during the first 24 h after adding the CM trio, but the amount of active phospho-p38 MAPK had risen 2.5-fold by 24 h (Fig. 9) when the phospho-MEK–ERKs were at their basal levels (Figs. 4 and 5). However, this rise was not maintained—the amount of phospho-p38 MAPK had dropped to the starting value by 72 h (Fig. 9) when the MEK–ERKs were active (Figs. 4–6).

DISCUSSION

To develop drugs to limit brain damage, such as that due to transient ischemia and the shower of ROS produced by reperfusion, we must find out how astrocytes induce the expression and activation of NOS-2 (also known as iNOS) because of the cytodestructive action of the excessive production of its NO product caused by proinflammatory cytokines. The road to excessive NO production begins with the stimulation of the gene encoding NOS-2.

There are different ways to get adult human astrocytes to express NOS-2. One way, is used in glaucoma. In this case, excessive strain from hydrostatic pressure triggers a biomechanical mechanism consisting of ligand-activated EGF receptors releasing NF-κB which travels to the nucleus where it binds to certain of its several binding sites in the NOS-2 gene's promoter [Taylor et al., 1998; Neufeld and Liu, 2003]. Another way, the one we have been studying here, is a cytokine-activated mechanism which also operates via NF-κB and requires the activation of p38 MAPK [Taylor et al., 1998; Neufeld and Liu, 2003]. Specifically, four NF-κB

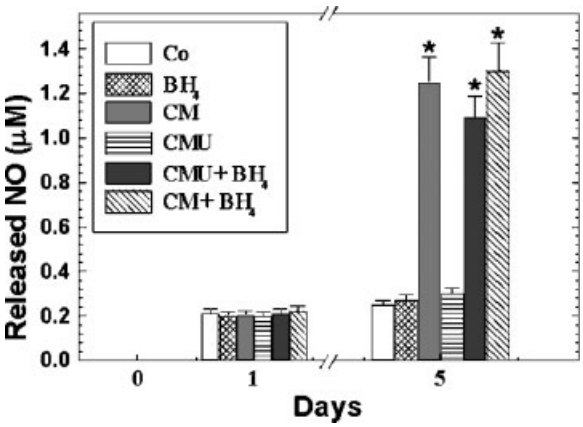

Fig. 8. Adding BH_4 (to a final concentration of 100 μM) enabled the NOS-2 protein accumulating in the astrocytes treated with the CM trio (IL-1β: 20 ng/ml, TNF-α: 20 ng/ml, and IFN-γ: 70 ng/ml) and two 30 min exposures to U0126 (at −0.5 and 0 h and 24–24.5 h) (CMU + BH_4 bar) to release nearly as much NO in their medium by 5 days as did the cells treated with the CM trio alone (CM bar). The concentrations of nitrate and nitrite derivatives of NO in the medium samples were measured to give the NO concentrations as described in ''Materials and Methods.'' None of the samples from the briefly U0126-treated cultures could have contained any U0126 as late as 4 days after the second and last exposure and washout. The bars are the mean ± SEM of the values from three separate experiments. The level of significance with respect to untreated controls was: $*P < 0.001$. Abbreviations: BH_4, tetrahydrobiopterin; Co, control; CM, CM trio-treated; CMU, CM, trio- and U0126-treated.

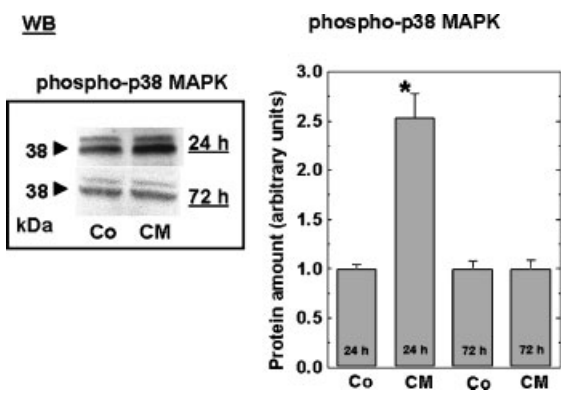

Fig. 9. The activation of p38 MAPK in astrocytes by 24 h after addition of the CM trio (IL-1β [20 ng/ml], TNF-α [20 ng/ml], and IFN-γ [70 ng/ml]) to their culture medium. However, the activity had dropped back to the basal level by 72 h when the MEK1/MEK2 and ERK1/ERK2 activities were at or near their peaks. Equal amounts of protein were extracted from CM trio-treated or untreated control (Co) astrocytes, subjected to SDS–PAGE electrophoresis, then transferred to nitrocellulose membranes and challenged with specific anti-phospho-p38 MAPK antibodies. The total p38 protein did not change (not shown). This immunoblot is typical of those from three separate experiments. The bars are the relative densities of the gels and are the mean ± SEM of the values from the three separate experiments. $*P < 0.001$ versus untreated (Co) astrocytes.

upstream enhancer elements in the NOS-2 gene promoter confer inducibility on the gene by IL-1β and TNF-α, but not IFN-γ [Taylor et al., 1998]. However, in some cells, such as our normal adult human astrocytes, the optimal activation of NOS-2 requires the cooperation with IL-1β and TNF-α or INF-γ which operates by activating STAT1α that travels to the nucleus where it binds to the NOS-2 gene's GAS (γ-interferon-activated site) site [Gao et al., 1997; Ohmori et al., 1997]. Thus, it is likely that the IL-1β + TNF-α + IFN-β trio used in our experiments started the chain of events leading to the production NO in our adult human astrocytes via a mixture of signals from three kinds of receptor that included an expected burst of p38 MAPK activity during the first 24 h (Fig. 9) that combined to drive NF-κB and STAT1 into the nucleus to stimulate the NOS-2 gene.

The first novel finding in this study is that the activation of the NOS-2 protein induced by the early CM trio-triggered events required MEK–ERK activity. Taking out MEK–ERKs with U0126 for the last 30 min before adding the CM triad did not at all affect the ultimate stimulation of NO production (Fig. 8). But the time when a 30 min exposure to U0126 could prevent NOS-2 activation and NO production was between 24 and 24.5 h when the U0126-insensitive p38 MAPK was at or near its peak (Figs. 7 and 9) and the MEK–ERK activities were still at their basal levels (Figs. 4 and 5). Therefore, it seems that there could have been another U0126-inhibitable enzyme—maybe the recently discovered MEK5–ERK5 [Kamakura et al., 1999]—operating at this time that was needed to start the MEK1/MEK2-ERK1/ERK2-activating and the NOS-2-activating mechanism. But again maybe not—U0126 did virtually eliminate the still basal phospho-MEK1/MEK2 activities in the CM trio-treated cells at 24.5 h (Fig. 4) which, though basal, might have been needed to maintain the responsiveness of the NOS-2 mechanism to the signals from the CM trio. This critical MEK1/MEK2-dependent process seems to have been available for starting NOS-2 activation only between 24 and 24.5 h after CM trio addition because it could not start again and drive NO production for at least 6.5 days after U0126 that had been added at 24h was washed out at 24.5 h (Fig. 7).

The second novel finding is that the U0126-sensitve process in the CM trio-stimulated

astrocytes is the synthesis of the NOS-2 co-factor BH_4, first because the U0126 prevents the buildup of BH_4 that accompanies the surge of NO production, and second because adding BH_4 to the culture medium enabled the U0126-treated cells to generate nearly normal amounts of NO (Fig. 7). Of course it has been known for some time that cytokines such as IFN-γ and TNF-α stimulate BH_4 synthesis [Werner et al., 1993, 1998, 2003], but to our knowledge this is the first indication of this action requiring MEK–ERK kinases. The BH_4 accumulating along with the new NOS-2 proteins in our CM trio-treated cells would have operated by binding with very high affinity to single NOS-2 pre-enzyme proteins and promoting their dimerization into functional enzymes [Werner et al., 1993, 1998, 2003; Tzeng et al., 1995; Thony et al., 2000]. Now we must try to find out in future experiments what happens during the critical 24–24.5 h to trigger BH_4 synthesis and how and what MEK–ERKs are involved in this process.

Finally the ability of U0126 to block NOS-2 activation and NO production in our adult astrocytes suggests that it and its relatives might be promising drugs for reducing damage in a stroked brain. NO from astrocytes stimulated by proinflammatory cytokines in the ischemic-reperfused brain could cause neighboring neurons to trigger apoptotic self destruction, and it appears that a stimulation of the MEK–ERK mechanism plays a major part in this reperfusion-NO-induced process in the neurons [Alessandrini et al., 1999; Namura et al., 2001]. Because of this, intracerebroventricular injection of PD98059 or intravenous injection of U0126 can as much as halve focal infarct volume in gerbil and mouse brains subjected to forebrain and focal cerebral ischemia [Alessandrini et al., 1999; Namura et al., 2001].

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REFERENCES

Alessandrini A, Namura S, Moskowitz MA, Bonventre JV. 1999. MEK1 protein kinase inhibition protects against damage resulting from focal cerebra ischemia. Proc Natl Acad Sci USA 96:12866–12869.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494.

Beach TG, Walker R, McGeer EG. 1989. Patterns of gliosis in Alzheimer's disease and aging cerebrum. Glia 2:420–436.

Bhat NR, Zhang P, Lee JC, Hogan EL. 1998. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 18:1633–1641.

Blank N, Burger R, Duerr B, Bakker F, Wohlfarth A, Dumitriu I, Kalden JR, Herrmann M. 2002. MEK inhibitor U0126 interferes with immunofluorescence analysis of apoptotic cell death. Cytometry 48:179–184.

Braet K, Cabooter L, Paemeleire K, Leybaert L. 2004. Calcium signal communication in the central nervous system. Biol Cell 96:79–91.

Choi HJ, Jang YJ, Kim HJ, Hwang O. 2000. Tetrahydrobiopterin is released from and causes preferential death of catecholaminergic cells by oxidative stress. Mol Pharmacol 58:633–640.

Da Silva J, Pierrat B, Mary JL, Lesslauer W. 1997. Blockade of p38 mitogen activated protein kinase pathway inhibits nitric oxide synthase expression in mouse astrocytes. J Biol Chem 272:28373–28380.

Davies SP, Reddy H, Caivano M, Cohen P. 2000. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95–105.

Dawson VL, Dawson TM. 1996. Nitric oxide in neural degeneration. Proc Soc Exp Biol Med 211:33–40.

Estévez A, Jordan J. 2002. Nitric oxide and superoxide, a deadly cocktail. Ann NY Acad Sci 962:207–211.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyke DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase. J Biol Chem 273: 18623–18632.

Fukushima T, Nixon JC. 1980. Analysis of reduced forms of biopterin in biological tissues and fluids. Anal Biochem 102:176–188.

Gao J, Morrison DC, Parmely TJ, Russell SW, Murphy WJ. 1997. An interferon-γ-activated site (GAS) is necessary for full expression of the mouse *iNOS* gene in response to interferon-γ and lipopolysaccharide. J Biol Chem 272: 1226–1230.

Grzybicki D, Moore SA, Schleper R, Glabinski AR, Ransohoff RM, Murphy S. 1998. Expression of monocyte chemoattractant protein (MCP-1) and nitric oxide synthase-2 following cerebral trauma. Acta Neuropathol (Berl) 95:98–103.

Hirsch EC, Breidert T, Rousselet E, Hunot S, Hartmann A, Michel PP. 2003. The role of glial reaction and inflammation in Parkinson's disease. Ann NY Acad Sci 991:214–228.

Itoh Y, Ma FH, Hoshi H, Oka M, Noda K, Ukai Y, Kojima H, Nagano T, Toda N. 2000. Determination and bioimaging method for nitric oxide in biological specimens by diaminofluorescein fluorometry. Anal Biochem 287:203–209.

Johnson GI, Lapadat R. 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK and p38 protein kinases. Science 298:1911–1912.

Kamakura S, Moriguchi T, Nishida E. 1999. Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. J Biol Chem 274:26563–26571.

Khaldi A, Chiueh CC, Bullock MR, Woodward JJ. 2002. The significance of nitric oxide production in the brain after injury. Ann NY Acad Sci 962:53–59.

Kojima H, Sakurai K, Kikuchi K. 1998. Development of a fluorescent indicator for nitric oxide based on the fluorescein chromophore. Chem Pharm Bull (Tokyo) 46: 373–375.

Leong S-L, Ruan R-S, Zhang Z. 2002. A critical assessment of the neurodestructive and neuroprotective effects of nitric oxide. Ann NY Acad Sci 962:161–181.

Lincoln J, Hoyle CHV, Burnstock G. 1997. Nitric oxide in health and disease. Cambridge: Cambridge University Press.

Liu B, Gao H-M, Wang J-Y, Jeohn G-H, Cooper CL, Hong J-S. 2002. Role of nitric oxide in inflammation-mediated neurodegeneration. Ann NY Acad Sci 962:318–331.

Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. 1993. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 214:11–16.

Namura S, Lihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, Moskowitz MA, Bonventre JA, Alessandrini A. 2001. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. Proc Natl Acad Sci USA 98:11569–11574.

Neufeld AH, Liu B. 2003. Comparison of the signal transduction pathways for the induction of gene expression of nitric oxide synthase-2 in response to two different stimuli. Nitric Oxide 8:95–102.

Norenberg MD. 1994. Astrocyte responses to CNS injury. J Neuropathol Exp Neurol 53:213–220.

Ohmori Y, Schreiber RD, Hamilton TA. 1997. Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κB. J Biol Chem 272:14899–14907.

Pearson G, Robinson F, Gibson TB, Xu B-E, Karandikar M, Berman K, Cobb MH. 2001. Mitogen-activated protein (MAP) kinase pathways: Regulation and phsyiological functions. Endocrine Rev 22:153–183.

Piao CS, Kim JB, Han PL, Lee JK. 2003. Administration of the p38 MAPK inhibitor SB203580 affords brain protection with a wide therapeutic window against focal ischemic insult. J Neurosci Res 73:537–544.

Ransom BR, Sontheimer HJ. 1992. The neurophysiology of glial cells. J Clin Neurophysiol 9:224–251.

Suzuki H, Menegazzi M, Carcereri De Prati A, Mariotto S, Armato U. 1995. Nitric oxide in the liver: Physiopathological roles. Adv Neuroimmunol 5:379–410.

Taylor BS, de Vera ME, Gunster RW, Wang Q, Shapiro RA, Morris SM, Jr., Billiar TR, Geller DA. 1998. Multiple NFκB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. J Biol Chem 273:15148–15156.

Thony B, Auerbach G, Blau N. 2000. Tetrahydrobiopterin biosynthesis, regeneration, and function. Biochem J 347: 1–16.

Tzeng E, Billiar TR, Robbins PD, Loftus M, Stuehr DJ. 1995. Expression of human inducible nitric oxide synthase in tetrahydrobiopterin (H4B) deficient cell line H4B promotes assembly of enzyme subunits into an active dimer. Proc Natl Acad Sci USA 92:11771–11775.

van Amsterdam JG, van den Berg C, Zuiderma J, te Biesbeek JD, Rokos H. 1996. Effect of septicaemia on the plasma levels of biopterin and nitric oxide in rats and rabbits. Biochem Pharmacol 52:1447–1451.

Wada K, Chatzipanteli K, Kraydieh S, Busto R, Dietrich WD. 1998. Inducible nitric oxide synthase expression after traumatic brain injury and neuroprotection with aminoguanidine treatment in rats. Neurosurgery 43: 1427–1436.

Werner ER, Werner-Felmayer G, Wachter H. 1993. Tetrahydrobiopterin and cytokines. Proc Soc Exp Biol Med 203:1–12.

Werner ER, Werner-Felmayer G, Mayer B. 1998. Tertrahydrobiopterin, cytokines, and nitric oxide synthesis. Proc Soc Exp Biol Med 219:171–182.

Werner ER, Gorren ACF, Heller R, Werner-Felmayer G, Mayer B. 2003. Tetrahydrobiopterin and nitric oxide: Mechanistic and pharmacological aspects. Proc Soc Exp Biol Med 228:1291–1302.

Xu X, Malave A. 2000. p38 MAPK, but not p42/p44 MAPK mediated inducible nitric oxide synthase expression in C6 glioma cells. Life Sci 67:3221–3230.

Zamora R, Vodovotz Y, Billiar TR. 2000. Inducible nitric oxide synthase and inflammatory diseases. Mol Med 6: 347–373.