

Methamphetamine Induces AP-1 and NF- κ B Binding and Transactivation in Human Brain Endothelial Cells

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Cellular and molecular mechanisms of methamphetamine (METH)-induced neurotoxicity may involve alterations of cellular redox status and induction of inflammatory genes in endothelial cells. To study these hypotheses, molecular signaling pathways of METH-induced inflammatory responses via activation of redox-sensitive transcription factors were investigated in human brain microvascular endothelial cells (HBMEC). A dose-dependent depletion of total glutathione levels was detected in HBMEC exposed to METH. In addition, electrophoretic mobility shift assay (EMSA) showed significant increases in DNA binding activities of redox-responsive transcription factors, AP-1 and NF- κ B, in HBMEC treated with METH. METH-mediated AP-1 or NF- κ B activation was accompanied by induction of transactivation of AP-1 or NF- κ B, as measured by dual luciferase assay using specific reporter plasmids. Because NF- κ B and AP-1 are known to regulate expression of inflammatory genes, expression of the gene encoding for tumor necrosis factor- α (TNF- α) was also studied in METH-treated HBMEC. A dose-dependent overexpression of the TNF- α gene was observed in HBMEC treated with METH. The importance of AP-1 and NF- κ B in METH-induced TNF- α gene was confirmed in functional promoter studies using constructs of the TNF- α promoter with mutated AP-1 or NF- κ B sites. These results indicate that METH-induced disturbances in cellular redox status and activation of AP-1 and NF- κ B can play critical roles in the signaling pathways leading to upregulation of inflammatory genes in human brain endothelial cells. *J. Neurosci. Res.* 66:583–591, 2001.

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Methamphetamine (METH) is a central nervous system stimulant of the amphetamine family. It causes dopaminergic neurotoxicity in mammals by producing long-term depletion of dopamine and its metabolites (Imam et al., 1999) and a decreased number of dopamine transporter binding sites (Wagner et al., 1980), as well as a reduction

in tyrosine hydroxylase and tryptophan hydroxylase activities (Hotchkiss and Gibb, 1980). A compelling body of evidence indicates that METH-induced neurotoxicity can be mediated by intracellular oxidative stress and free radical generation. For example, Yamamoto and Zhu (1998) suggested that METH can increase production of reactive oxygen species and Imam et al. (1999) demonstrated that peroxynitrite can play a role in METH-induced dopaminergic neurotoxicity in mice. Moreover, administration of antioxidants and spin trapping agents attenuated METH-induced toxicity (DeVito and Wagner, 1989; Cappon et al., 1996; Yamamoto and Zhu, 1998; Imam and Ali, 2000). To support these observations, it was reported that METH-induced toxicity was attenuated in transgenic mice that overexpressed copper-zinc superoxide dismutase (Cadet et al., 1994). Even though these reports indicated that oxidative stress can be implicated in METH-induced cell injury, cellular and molecular mechanisms underlying this cytotoxicity are not fully understood. Specifically, there are no reports on METH-induced effects on brain endothelial cells.

Recent evidence indicates the critical role of intracellular redox status in regulatory pathways leading to activation of a variety of transcription factors and gene expression. It appears that among compounds that can influence cellular redox potential, glutathione plays one of the most important roles. Indeed, intracellular glutathione is the major non-protein sulfhydryl compound that can control the regeneration of protein sulfhydryl groups and thus regulate intracellular redox status (Sies, 1999).

Activator protein-1 (AP-1) and nuclear factor- κ B (NF- κ B) belong to a family of transcription factors whose activity is regulated by cellular redox status (Schreck et al., 1992; Xanthoudakis et al., 1992; Bouloumie et al., 1999). In addition, these transcription factors can regulate expres-

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sion of the inflammatory genes, such as gene encoding for tumor necrosis factor- α (TNF- α) (Pinkus et al., 1996; Dalton et al., 1999). Indeed, the TNF- α gene was shown to be regulated by redox mechanisms at the transcription level (Rahman and MacNee, 2000; Verhasselt et al., 1998). To support this observation, Guha et al. (2000) demonstrated that activation of transcription factors AP-1 and NF- κ B plays the critical role in enhanced TNF- α mRNA transcription.

The aim of the present study was to investigate the molecular signaling pathways of METH-induced alterations of cellular redox status in human brain microvascular endothelial cells (HBMEC). We demonstrate that exposure of HBMEC to METH can markedly activate AP-1 and NF- κ B as well as stimulate TNF- α gene transcription through AP-1 and NF- κ B-dependent pathways.

MATERIALS AND METHODS

Cell Culture

Human brain microvascular endothelial cells (HBMEC) were isolated, cultivated and purified as previously described (Stins et al., 1997). These cells were positive for factor VIII-Rag, carbonic anhydrase IV, Ulex Europeus Agglutinin I, took up fluorescently labeled low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain endothelial cell characteristics. Contamination of non-endothelial cells such as pericytes and glial cells were less than 1%. HBMEC were cultured in RPMI 1640-based medium with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 10% NuSerum (Becton Dickinson, Bedford, MA), 30 μ g/ml of endothelial cell growth supplement (ECGS, Becton Dickinson), 15 U/ml of heparin, 2 mM L-glutamine, 2 mM sodium pyruvate, nonessential amino acids, vitamins, 100 U/ml of penicillin, and 100 μ g/ml of streptomycin (all reagents, Gibco BRL, Grand Island, NY). Vitamin mixture did not contain vitamins E and C. Cultures were incubated at 37°C in a humid atmosphere of 5% CO₂. Each experiment was carried out at least three times, and each time on a different day.

Glutathione Determination

Total glutathione was determined using the microtiter plate assay method of Baker et al. (1990) with slight modifications. HBMEC, incubated in the absence or presence of METH for 3 hr at 37°C, were washed with phosphate-buffered saline and pelleted at 4°C. Cellular glutathione was extracted in 2.25% 5-sulfosalicylic acid. After centrifugation at 14,000 \times *g* for 20 min at 4°C, the supernatant was used for the determination of total glutathione, whereas the pellet was dissolved in 0.2 M NaOH solution containing 0.1% SDS for protein concentration analysis. Levels of total glutathione in acid-soluble fractions were determined by enzymatic recycling assay in the presence of 0.15 mM 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), 0.2 mM NADPH, and 1.0 U of glutathione reductase/ml of assay mixture in 0.3 mM EDTA, 30 mM sodium phosphate buffer, pH 7.5. Total glutathione was estimated by monitoring the rate of formation of chromophoric product 2-nitro-5-thiobenzoic acid at 405 nm. The glutathione content in samples was calculated on the basis of the standard curve obtained with known amounts of

glutathione. Protein concentration was determined as described by Bradford (1976) using albumin as a standard. Data are expressed as nmol of glutathione per mg of protein.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts from HBMEC were prepared as described (Beg et al., 1993). Double-stranded oligonucleotides containing the consensus sequence of the binding site for transcription factor AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3' or NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG-3') were purchased from Promega (Madison, WI) and labeled with [γ -³²P]-ATP (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase. The reaction mixture was incubated for 1 hr at 37°C. After incubation, T4 polynucleotide kinase was inactivated by placing the tube for 10 min at 68°C on a heat block. Unincorporated nucleotides were removed by gel filtration chromatography using mini Quick Spin Oligo Columns (Boehringer-Mannheim Corporation, Indianapolis, IN). Binding reactions were carried out in a 20 μ l vol containing 4 μ g of nuclear protein extracts, 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol, 10% glycerol, 2 μ g of poly[dl-dC] (nonspecific competitor) and 40,000 cpm of ³²P-labeled specific oligonucleotide probe. For supershift experiments, 4 μ g of nuclear protein extracts were incubated for 25 min at room temperature with 4 μ g of specific antibody (rabbit polyclonal anti-c-Jun, goat polyclonal anti-c-Fos, goat polyclonal anti-NF- κ B p50, or rabbit polyclonal anti-NF- κ B p65, Santa Cruz Biotechnology, Santa Cruz, CA) in the presence of binding buffer and 2 μ g of poly[dl-dC]. Then, 40,000 cpm of ³²P-labeled specific oligonucleotide probe was added and the mixture (20 μ l of total vol) was incubated for 25 min at room temperature. Resultant protein-DNA complexes were resolved on native 5% polyacrylamide gels using 0.25 \times TBE buffer (50 mM Tris-Cl, 45 mM boric acid, 0.5 mM EDTA, pH 8.4). Competition studies were carried out by the addition of a molar excess of unlabeled oligonucleotide to the binding reaction.

Transfection and Dual Luciferase Assays

Transient transfections of HBMEC were carried out using pF_x-7 (Invitrogen, Carlsbad, CA) as described earlier (Kaiser and Toborek, 2001) with modification. Cells were transfected with 10 μ g of the firefly luciferase reporter plasmids and co-transfected with 0.5 μ g of the control *Renilla* luciferase vector (pRL-SV40; Promega). Although the pRL-SV40 vector contains NF- κ B and AP-1 binding sites, preliminary studies indicated that treatments with METH did not upregulate *Renilla* luciferase activity in endothelial cells co-transfected with this vector. Therefore, the pRL-SV40 vector could be used as a control vector to normalize transfection rates in cultured endothelial cells. Firefly luciferase reporter plasmids employed in the present study included AP-1- and NF- κ B-responsive constructs [p(AP-1)Luc and p(NF- κ B)Luc, respectively; Stratagene, La Jolla, CA], a construct of normal TNF- α promoter [pTNF(-615)Luc], as well as constructs of the TNF- α promoter with mutated AP-1 or NF- κ B binding sites [pTNF(mAP-1)Luc and pTNF(mNF- κ B₃)Luc, respectively]. Generation of the TNF- α promoter constructs was described earlier (Yao et al., 1997). After transfection, cultures were maintained in normal growth

medium for 24 hr and then exposed to methamphetamine for an additional 16 hr in RPMI 1640 media. Determination of firefly and *Renilla* luciferase activities were carried out using the Dual-Luciferase[®] Reporter Assay System (Promega). Briefly, the cells were washed with phosphate-buffered saline and lysed with Passive Lysis Buffer. Cell lysates were mixed with Luciferase Assay Reagent II, and the firefly luminescence was measured using a luminometer with dual automatic injector (Turner Designs, CA). Next, samples were mixed with the Stop and Glo reagent and the *Renilla* luciferase activity was measured as an internal control. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted by the use of TRI reagent (Sigma Chemical Company, St. Louis, MO) and reverse-transcribed at 42°C for 60 min in 20 μ l of 5 mM MgCl₂, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/ μ l of recombinant RNasin ribonuclease inhibitor, 15 U/ μ g of AMV reverse transcriptase, and 0.5 μ g of oligo(dT)₁₅ primer. For amplification of the TNF- α and of the β -actin (a housekeeping gene), the following primer combinations were used: 5'-GTGACAAGCCTGTAGCCCA-3' and 5'-ACTCGGCAAAGTTCGAGATAG-3' (TNF- α ; expecting 414-bp fragment) (R&D Systems, Inc., Minneapolis, MN), and 5'-AGCACAATGAAGATCAAGAT-3' and 5'-TGTAACGCAACTAAGTCATA-3' (β -actin; expecting 188-bp fragment) (Ballester et al., 1998). The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA), 2 μ l of the reverse transcriptase reaction, and 20 pmol of primer pairs in a total vol of 50 μ l. Thermocycling was carried out according to the following profile: 94°C for 1 min before the first cycle, 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec, repeated 25 times and followed by a final extension at 72°C for 10 min. Amplification was linear within the range of 20–35 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR[®] Green I (Molecular Probes, Eugene, OR) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN).

Statistical Analysis

Routine statistical analysis of data was completed using SYSTAT 7.0 (SPSS Inc., Chicago, IL). One-way ANOVA was used to compare mean responses among the treatments. The treatment means were compared using Bonferroni least significant difference procedure. Statistical probability of $P < 0.05$ was considered significant.

RESULTS

METH Decreases Total Glutathione Levels in Brain Endothelial Cells

Involvement of the glutathione antioxidant system is a characteristic feature of intracellular oxidative stress (Moszczynska et al., 1998). Therefore, we determined levels of total glutathione in HBMEC by enzymatic recycling assay using glutathione reductase and 5,5'-dithio-bis-2-nitrobenzoic acid. Figure 1 shows the effect of METH

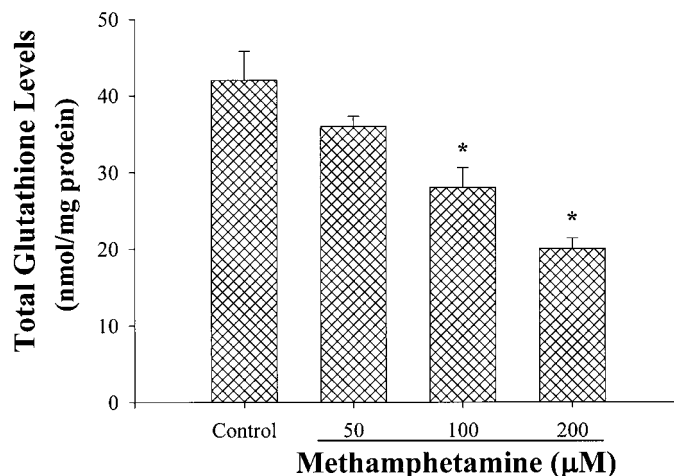


Fig. 1. Methamphetamine decreases intracellular total glutathione levels in human brain microvascular endothelial cells (HBMEC). HBMEC were exposed to increasing doses of METH for 3 hr. Values represent mean \pm SD. *Statistically significant as compared to control cultures ($P < 0.05$).

on total glutathione concentration in cultured endothelial cells. Treatment with METH for 3 hr resulted in a significant depletion of cellular glutathione levels in a dose-dependent manner. In HBMEC treated with 200 μ M METH, the decrease in total glutathione levels was approximately 50%. In addition, statistically significant diminished glutathione concentration was observed in cells exposed for 3 hr to 100 μ M METH. In contrast, glutathione levels were within the control range in cells treated with METH for 24 hr (data not shown). Therefore, it appears that the initial decrease in glutathione levels in METH-treated HBMEC is reversible after a prolonged exposure to this drug.

METH Activates AP-1 Binding and Transactivation in Brain Endothelial Cells

Electrophoretic mobility shift assay was carried out to investigate DNA binding activities of the redox-responsive transcription factors AP-1 and NF- κ B in HBMEC exposed to METH. Nuclear extracts of cells treated with METH for 2 hr showed a dose-dependent induction of the AP-1 binding activity (Fig. 2A, lane 3–5). METH at the doses of 100 and 200 μ M strongly activated AP-1 binding. The specificity of the AP-1 binding was determined by competition experiments with molar excess of unlabeled oligonucleotide containing the AP-1 binding site (Fig. 2A, lane 6) and supershift analysis with antibodies against c-Jun and c-Fos (Fig. 2B). Incubation of nuclear extracts with anti-Jun antibody resulted in a decrease in intensity of the AP-1 band by approximately 30% as revealed by densitometric analysis (Fig. 2B, lane 3). In contrast, incubation with anti-Fos antibody produced supershift of the AP-1 band (Fig. 2B, lane 4). To ensure that the effects of anti-Jun and anti-Fos antibodies are specific, nuclear extracts isolated from METH-treated cells were

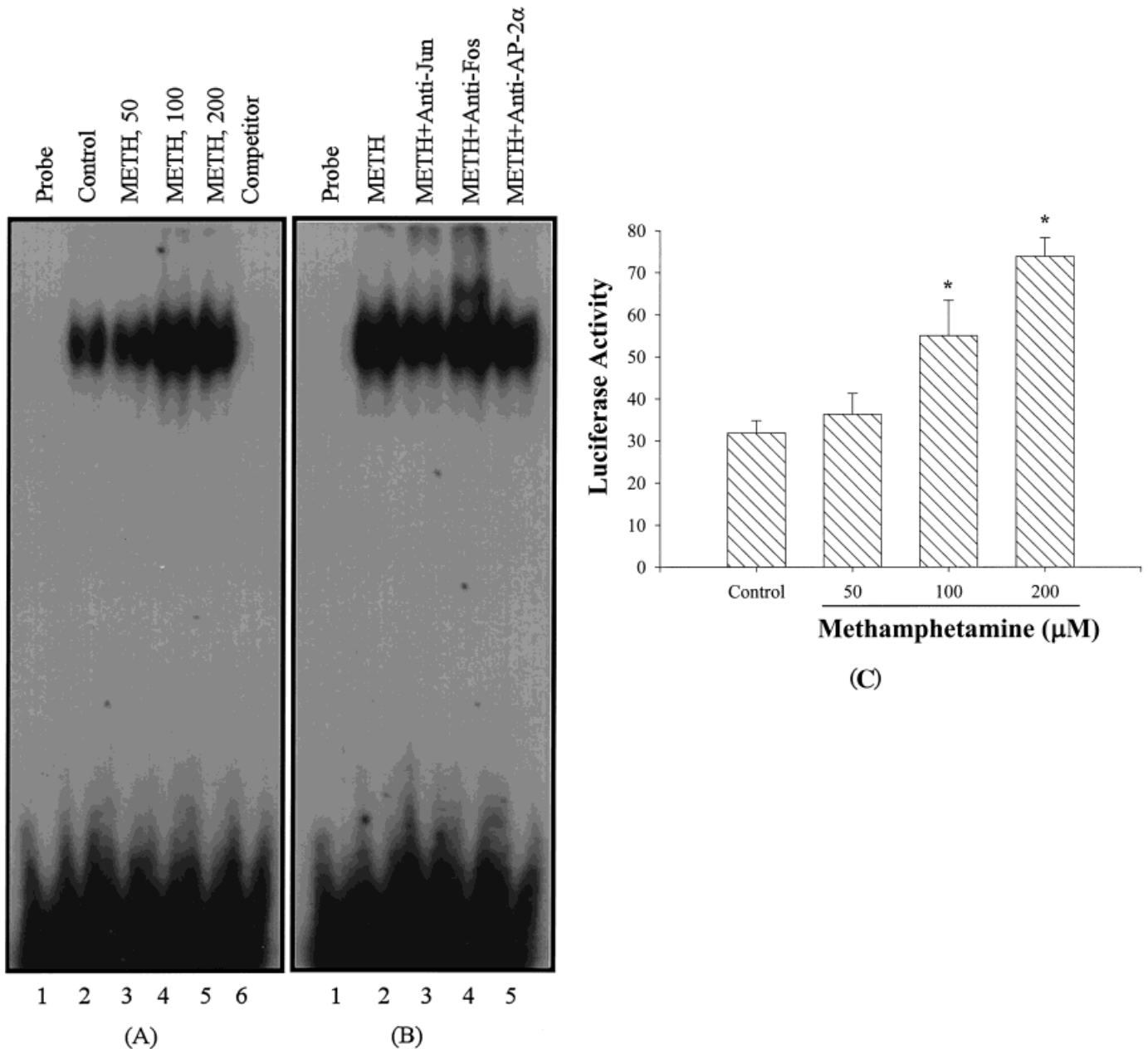


Fig. 2. **A:** Methamphetamine enhances binding activity of AP-1 in human brain microvascular endothelial cells (HBMEC). HBMEC were either untreated (**lane 2**, control cultures) or treated with the indicated doses of methamphetamine (**lanes 3–5**) for 2 hr. Nuclear extracts were prepared and analyzed by EMSA. Competition study was carried out by the addition of excess unlabeled oligonucleotide (**lane 6**) using nuclear extract from cells stimulated by 200 μ M methamphetamine. **B:** Supershift analysis of AP-1 binding activity. Nuclear extracts were prepared from cells treated with 200 μ M methamphetamine for 2 hr (lanes 2–5)

and incubated with anti-AP-2 α antibody. The AP-2 α binding site does not overlap with either AP-1 or NF- κ B binding sites. As indicated in Figure 2B (lane 5), anti-AP-2 α antibody did not have an apparent effect on AP-1 binding reactions.

and incubated with anti-c-Jun antibody (lane 3), anti-c-Fos antibody (lane 4), or anti-AP-2 α antibody (lane 5) for 25 min before the addition of 32 P-labeled probe; **lane 1**, probe alone. **C:** Methamphetamine increases AP-1 transactivation in human brain microvascular endothelial cells (HBMEC) as measured by dual luciferase assay. The cells were transfected with the p(AP-1)Luc plasmid and co-transfected with the internal *Renilla* luciferase control vector (pRL-SV40) to normalize transfection rates. Values represent mean \pm SD. *Statistically significant as compared to the untreated control cultures ($P < 0.05$).

To determine the ability of METH-mediated activation of AP-1 to induce gene expression, HBMEC were transfected with luciferase reporter plasmid containing direct repeats of AP-1 consensus sequence [p(AP-1)Luc]. As shown in Figure 2C, treatment with 100 or 200 μ M

METH produced significant and dose-dependent elevation of luciferase activity. These data indicate that METH-induced AP-1 activation is sufficient to induce AP-1-dependent transcription in HBMEC.

METH Activates NF- κ B Binding and Transactivation in Brain Endothelial Cells

Similar to AP-1, a significant increase in NF- κ B binding activity was detected in nuclear extracts prepared from HBMEC treated with increasing doses of METH (Fig. 3A, lane 3–5). Treatment with METH at the concentration of 50 μ M induced only a slight activation of NF- κ B. Exposure to this drug at the concentration of 100 and 200 μ M, however, resulted in more marked NF- κ B activation. The identity of the NF- κ B binding was confirmed by competition binding with the molar excess of unlabeled NF- κ B probe (Fig. 3A, lane 6) and supershift analysis with the specific anti-p50 or anti-p65 antibodies (Fig. 3B, lanes 3 and 4). Specific effects of anti-p50 and anti-p65 antibodies were further demonstrated in the reaction in which nuclear extracts isolated from METH-treated cells were incubated with anti-AP-2 α antibody. As indicated in Figure 3B (lane 5), anti-AP-2 α antibody did not alter NF- κ B binding reaction.

To determine whether METH-induced activation of NF- κ B can induce gene expression, NF- κ B-dependent transcription was measured in HBMEC transfected with luciferase reporter plasmid containing direct repeats of NF- κ B consensus sequence [p(NF- κ B)Luc]. As indicated in Figure 3C, METH-mediated activation of NF- κ B was sufficient to induce transactivation of NF- κ B. As compared to control cultures, NF- κ B-regulated luciferase activity was higher in HBMEC treated with 100 μ M or 200 μ M METH.

METH Induces TNF- α Gene Expression in Brain Endothelial Cells Through AP-1 and NF- κ B-Dependent Pathways

TNF- α is a potent inflammatory cytokine and the TNF- α gene promoter contains binding sites for AP-1 and NF- κ B. In addition, it was shown that the expression of the TNF- α gene is dependent on activation of AP-1 and NF- κ B. Therefore, induction of the TNF- α gene was determined in HBMEC treated with METH for 2 hr. As indicated in Figure 4A, METH induced a dose-dependent upregulation of TNF- α gene expression. This effect was seen in HBMEC treated with doses of METH as low as 25 μ M.

To prove that the AP-1 and NF- κ B binding sites play critical roles in METH-induced TNF- α expression in HBMEC, cells were transfected either with the luciferase construct of normal TNF- α promoter [pTNF(-615)Luc] or with constructs of the TNF- α promoter with mutated either the AP-1 site [pTNF(mAP-1)Luc] or the NF- κ B site [pTNF(mNF- κ B₃)Luc]. In addition, HBMEC were co-transfected with the *Renilla* luciferase control vector (pRL-SV40) to normalize transfection rates. As indicated in Figure 4B, exposure to METH induced luciferase activity only in cells transfected with construct of the normal

TNF- α promoter. In contrast, mutations in AP-1 or NF- κ B sites completely inhibited METH-induced stimulation of luciferase activity in transfected HBMEC.

DISCUSSION

Brain microvascular endothelial cells are responsible in large part for the formation and function of the blood-brain barrier (BBB) (Staddon et al., 1995). These cells form an uninterrupted capillary wall providing the brain with a selective transport mechanism. In addition, BMEC are surrounded by processes of the perivascular astroglia that contribute to the formation of the BBB (Rubin and Staddon, 1999). Evidence indicates that metabolism of brain microvascular endothelial cells and functions of the BBB can be modulated by environmental stimuli through generation of reactive oxygen species, alterations of endothelial cell redox status and disruption of the BBB integrity (Mayhan, 1995; Stanimirovic et al., 1995).

Results of the current study demonstrate that METH can induce alterations of cellular redox status in HBMEC. This notion is supported by the observation that a dose-dependent depletion of total glutathione levels was detected in HBMEC exposed to METH. These results are in line with earlier reports that implicated disturbances in glutathione metabolism in METH-induced neurotoxicity (Cadet and Brannock, 1998). For example, Moszczynska et al. (1998) reported a significant reduction in total glutathione in METH-administered rat striatum, and Harold et al. (2000) suggested that METH could alter the glutathione system in rat striatum in response to oxidative stress. It should be noted that the cellular glutathione redox system is the critical part of the endothelial cell antioxidant system, and depletion of glutathione appears to be a common cellular response to increased oxidative stress (Toborek et al., 1995; Hennig et al., 1996).

The observed METH-induced decrease in glutathione within the first 3 hr of exposure was reversed in HBMEC treated with this drug for 24 hr. Such reversible changes in glutathione levels are frequently observed in cultured endothelial cells exposed to pro-oxidant stimuli. For example, we showed an initial decrease in glutathione levels in peripheral endothelial cells exposed to selected free fatty acids for 3–6 hr, which was followed by an increase in later time points (Toborek and Hennig, 1994; Toborek et al., 1996). The enhanced glutathione levels after a prolonged exposure to pro-oxidants can be part of a general antioxidant protection. The rapid glutathione synthesis and short turnover of utilizable glutathione, approximately 5 hr in endothelial cells (Cotgreave et al., 1991), make possible a rapid replacement of the cellular glutathione pool. On the other hand, the decrease in glutathione level observed within the first 3 hr of METH treatment appears to be sufficient to initiate activation of redox-regulated transcription factors with subsequent induction of the inflammatory genes, such as the TNF- α gene.

Induction of cellular oxidative stress or changes of intracellular glutathione levels can trigger signal transduction pathways via activation of redox-responsive transcrip-

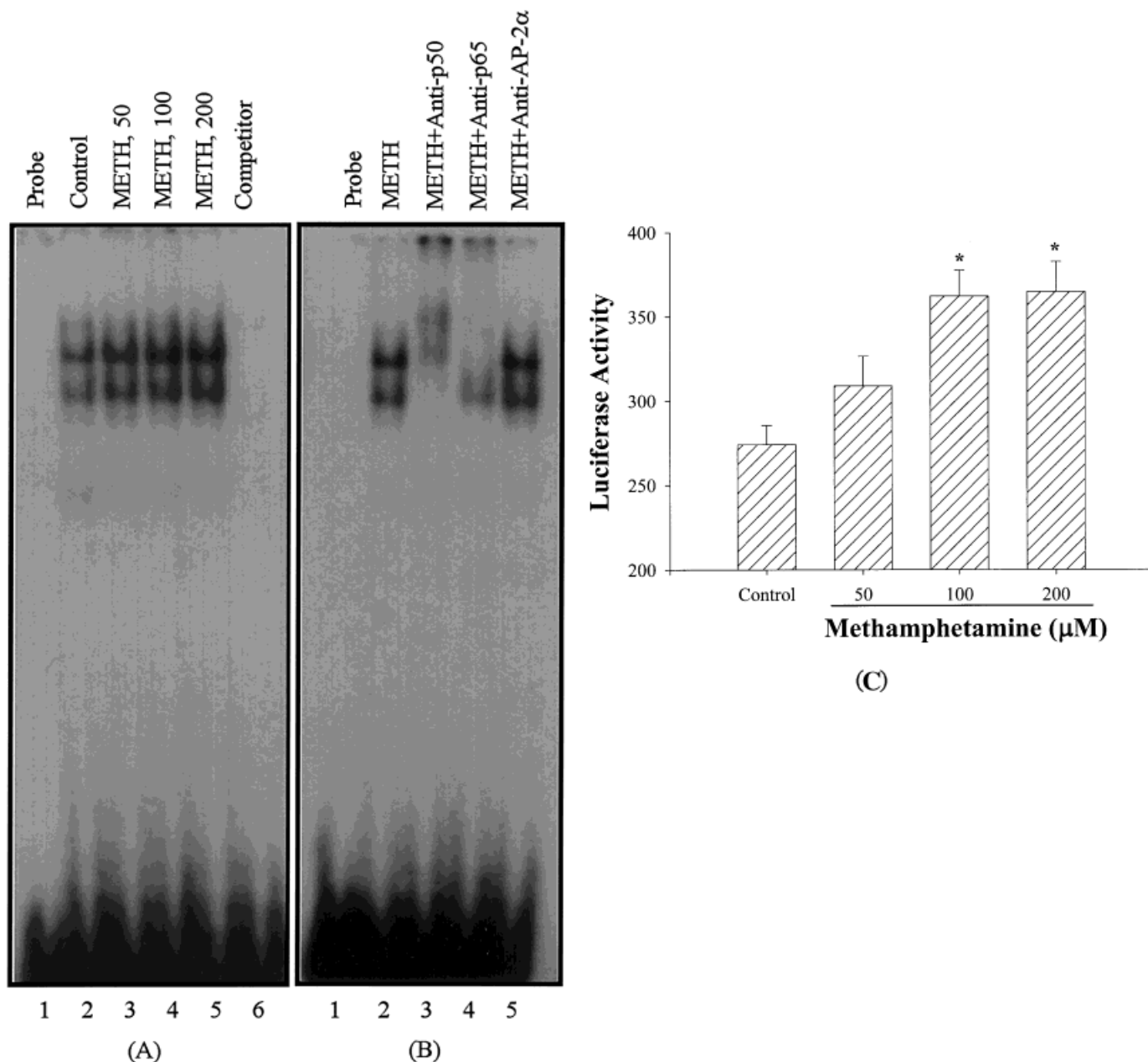


Fig. 3. **A:** Methamphetamine enhances binding activity of NF- κ B in human brain microvascular endothelial cells (HBMEC). HBMEC were either untreated (**lane 2**, control cultures) or treated with the indicated doses of methamphetamine (**lanes 3–5**) for 2 hr. Nuclear extracts were prepared and analyzed by EMSA. Competition study was carried out by the addition of excess unlabeled oligonucleotide (**lane 6**) using nuclear extract from cells stimulated by 200 μ M methamphetamine. **B:** Supershift analysis of NF- κ B binding activity. Nuclear extracts were prepared from cells treated with 200 μ M methamphetamine for 2 hr (lanes 2–5)

and incubated with anti-p50 antibody (lane 3), anti-p65 antibody (lane 4), or anti-AP-2 α antibody (lane 5) for 25 min before the addition of 32 P-labeled probe; **lane 1**, probe alone. **C:** Methamphetamine increases NF- κ B transactivation in human brain microvascular endothelial cells (HBMEC) as measured by dual luciferase assay. HBMEC were transfected with the p(NF- κ B) Luc plasmid and co-transfected with the internal *Renilla* luciferase control vector (pRL-SV40) to normalize transfection rates. Values represent mean \pm SD. *Statistically significant as compared to control cultures ($P < 0.05$).

tion factors and, hence, the transcription of specific genes. Indeed, transcription factors AP-1 and NF- κ B have been known to be activated in response to alterations of cellular redox status in a wide range of cells, leading to the up-

regulation of a number of proinflammatory genes (Arrigo et al., 1999; Bouloumie et al., 1999). AP-1 is a family of basic domain/leucine zipper transcription factors that have been characterized for the specific binding to and trans-

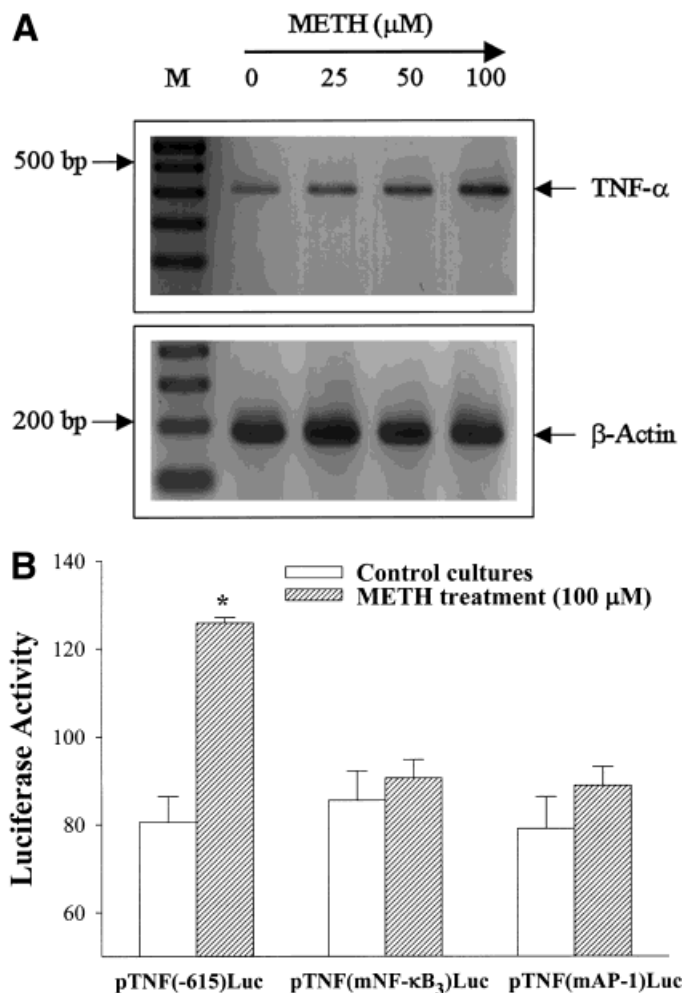


Fig. 4. **A:** Methamphetamine (METH) upregulates the TNF- α mRNA expression in human brain microvascular endothelial cells (HBMEC). HBMEC were exposed to increasing doses of methamphetamine for 2 hr. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphorimaging. The predicted sizes of RT-PCR products for TNF- α and β -actin (represented by arrows) are 414 bp and 188 bp, respectively. M, molecular weight markers (100 bp DNA ladder). **B:** Functional analysis of the AP-1 and NF- κ B₃ binding sites of the human TNF- α promoter in methamphetamine (METH)-treated human brain microvascular endothelial cells (HBMEC). HBMEC were transfected with the construct of normal TNF- α promoter [pTNF(-615)Luc] as well as constructs of the TNF- α promoter with mutated AP-1 or NF- κ B binding sites [pTNF(mAP-1)Luc and pTNF(mNF- κ B₃)Luc, respectively] and either untreated or treated with 100 μ M METH for 16 hr. Mutation of the AP-1 or NF- κ B site in the TNF- α promoter construct completely inhibited METH-induced luciferase activity. *Values in the group treated with METH are statistically significant as compared to the untreated control ($P < 0.05$).

activation through a cis-acting 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) response element. AP-1 is composed of the Jun and Fos gene products, which can form heterodimers (Jun/Fos) or homodimers (Jun/Jun).

Mechanisms of AP-1 activation are not fully understood. For example, it was shown that under specific experimental conditions, both oxidants and antioxidants can lead to activation of this transcription factor (for reviews see Gius et al., 1999, Hsu et al., 2000). For example, it was shown that c-Fos/c-Jun binding activity toward AP-1 sites is regulated by oxidative status of cysteine residues of c-Fos and c-Jun proteins (Fos Cys-154 and Jun Cys-272, respectively). Oxidation of cysteine residues can convert c-Fos or c-Jun into inactive forms. In contrast, a reduction of these residues can re-activate c-Fos/c-Jun binding activity (Xanthoudakis et al., 1992). On the other hand, oxidative stress also can induce mitogen-activated protein kinase (MAPK) cascade that, in subsequent reactions, can lead to AP-1 activation. In fact, the MAPK pathway is considered to be a major signal transduction mechanism leading to phosphorylation and activation of c-Fos (Hill and Treisman, 1995), and METH can induce MAPK activity (Hebert and O'Callaghan, 2000). To support the role of AP-1 in METH-induced cellular activation and toxicity, it was demonstrated that METH can induce AP-1 DNA-binding activity in mice (Sheng et al., 1996a). Detailed mechanisms of METH-induced activation of AP-1, however, remain to be determined.

NF- κ B is a ubiquitously expressed multisubunit transcription factor that is activated by oxidative stress produced by extracellular stimuli such as TNF- α , IL-1 β , and lipopolysaccharide (LPS) and inhibited by the antioxidants pyrrolidine dithiocarbamate (PDTTC) and N-acetylcysteine (NAC) (Schreck et al., 1992). NF- κ B is composed of homo- or heterodimeric complexes of at least five distinct subunits, such as p50, p52, p65 (RelA), c-Rel, and Rel-B; however, the p50/p65 heterodimer is the predominant form of this transcription factor (Baeuerle, 1998). In the present study, the composition of active NF- κ B p50/p65 heterodimer in METH-treated HBMEC was confirmed using supershift assays with specific anti-p50 and anti-p65 antibodies (Fig. 3B). It is well known that activation of NF- κ B is strongly regulated by cellular redox status and, thus, by intracellular glutathione levels (Droge et al., 1994). Therefore, a METH-induced decrease in the concentration of cellular glutathione could be responsible for activation of NF- κ B as observed in the present study (Fig. 3). To support the role of METH in NF- κ B activation, it was demonstrated that METH injections can activate NF- κ B DNA-binding in the striatum in mice (Asanuma and Cadet, 1998).

To determine whether METH-mediated activation of AP-1 and NF- κ B can lead to expression of genes regulated by these transcription factors, we determined effects of METH on transactivation of AP-1 or NF- κ B as measured by reporter gene assay in HBMEC transfected with the p(AP-1)Luc or the p(NF- κ B)Luc. Indeed, activation of AP-1 or NF- κ B by METH in HBMEC was associated with AP-1- or NF- κ B-dependent transcription. These findings were confirmed in experiments in which expression of the TNF- α gene was studied in HBMEC exposed to METH. AP-1 and NF- κ B are critical tran-

scription factors in the regulation of the TNF- α gene, and, as indicated in the present study, treatment with METH induced a dose-dependent increase in TNF- α mRNA levels. In addition, in HBMEC transfected with the TNF- α promoter constructs with mutated AP-1 or NF- κ B sites, no gene expression was observed (Fig. 4). It should be noted that the present report provides the first evidence that METH can induce TNF- α gene expression in HBMEC. TNF- α is recognized to be an important mediator of inflammatory events in the central nervous system (Shi et al., 1998). In addition, elevated TNF- α levels can increase endothelial cell permeability. We (Toborek et al., 1995) and others (Goldblum et al., 1993) demonstrated alteration of barrier function in peripheral endothelial cells exposed to TNF- α . Therefore, overexpression of the TNF- α gene can contribute to METH-induced cellular injury and degeneration.

Although current studies focused on METH-induced activation of redox-regulated transcription factors, such as NF- κ B and AP-1, evidence indicates that this abused drug also may induce alterations in brain vasculature due to other mechanisms. For example, injections with METH were shown to induce cyclooxygenase-2 (COX-2), a potent proinflammatory enzyme (Kita et al., 2000). In addition, METH abuse is known to be associated with the risk for brain hemorrhages. It was determined that acute intoxication with METH may result in formation and rupture of a berry aneurysms with subsequent subarachnoid and intracerebral hemorrhages (Davis et al., 1996; Karch et al., 1999). These effects, however, were rather linked to METH-induced transient hypertension and tachycardia and were not considered to be caused by the direct influence of METH on brain vasculature (Davis et al., 1996). Finally, it is not clear how hypothermic effects of METH (Yu et al., 1999) can affect vascular, and specifically, endothelial cell inflammatory responses.

In conclusion, to our knowledge, the present study is the first report to show the direct effects of METH on human brain endothelial cells. Exposure of HBMEC to METH can induce alterations of cellular redox status manifested by decreased total glutathione content. Such alterations can be responsible for METH-mediated activation of oxidative stress-responsive transcription factors, such as NF- κ B and AP-1, followed by upregulation of the inflammatory genes, such as the TNF- α gene. Upregulation of the inflammatory genes may contribute to METH-mediated induction of inflammatory processes in the central nervous system and participate in the breakdown of the BBB.

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