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The Increase in TNF-α Levels Is Implicated in NF-κB Activation and Inducible Nitric Oxide Synthase Expression in Brain Cortex after Immobilization Stress

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The underlying mechanisms by which physical or psychological stress causes neurodegeneration are still unknown. We have demonstrated that the high-output and long-lasting synthesizing source of nitric oxide (NO), inducible NO synthase (iNOS), is expressed in brain cortex after three weeks of repeated stress and that its overexpression accounts for the neurodegenerative changes found in this situation. Now we have found that a short duration of stress (immobilization for 6 h) also induces the expression of iNOS in brain cortex in adult male rats. In order to elucidate the possible mechanisms involved in iNOS expression, we have studied the role of the cytokine tumor necrosis factor-α (TNF-α) released in brain during stress. We have shown that there is an increase in soluble TNF-α levels after 1 h of stress in cortex and that this is preceded by an increase in TNF-α-convertase (TACE) activity in brain cortex as soon as 30 min after immobilization. Stress-induced increase in both TACE activity and TNF-α levels seems to be mediated by excitatory amino acids since they can be blocked by MK-801 (dizocilpine) (0.2 mg/kg i.p.), an antagonist of the N-methyl-D-aspartate subtype of glutamate receptor. In order to study the role of TACE and TNF-α in iNOS induction, a group of animals were i.p. injected with the preferred TACE inhibitor BB1101 (2 and 10 mg/kg). Indeed, BB1101 inhibited iNOS expression induced by six hours of stress. In addition, we studied the role of the transcription factor nuclear factor κB (NF-κB), which is required for iNOS expression. We have found that the administration of the TACE inhibitor BB1101 inhibited the stress-stimulated translocation of NF-κB to the nucleus. Taken together, these findings indicate that glutamate receptor activation induces TACE up-regulation and subsequent increase in TNF-α levels, and this account for stress-induced iNOS expression via NF-κB activation, supporting a possible neuroprotective role for specific TACE inhibitors in this situation.

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Inducible nitric oxide synthase (iNOS) is a high-output isoform of NOS which has been implicated in cellular toxicity in many cell systems including brain. Nitric oxide, when generated in high quantities following induction of iNOS, combines with the superoxide anion to form highly reactive, death-inducing compounds such

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as peroxynitrite (Moncada et al. 1991; Gross and Wolin 1995). In this context, we have demonstrated that restrain stress induces a generalized increase in NO production (Leza et al. 1998) and that iNOS is expressed in brain cortex of rats exposed to stress (Olivenza et al. 2000). In support of the idea that iNOS is one of the mechanisms responsible for the functional damage in this condition, we have also demonstrated that aminoguanidine, a preferred inhibitor of iNOS, protects against cell damage produced by immobilization stress in rats (Olivenza et al. 2000; Madrigal et al. 2000), an experimental procedure used as a model of stress disorders in humans (Bremner et al. 1991).

These evidences indicate that stress-induced iNOS expression in brain may mediate, at least in part, the anatomical and clinical features of neurotoxic damage found in animals and humans after exposure to uncontrollable stress (Sheline et al. 1996; Sapolsky 1996; Kim and Yoon 1998). This emphasizes the interest of the study of the mechanisms of iNOS expression after stress exposure. iNOS, as an inducible protein, is mainly regulated at the transcriptional level and is expressed after exposure of cells to several noxious agents such as cytokines and/or lipopolysaccharide (rev. in Nathan and Xie 1994). Although a considerable amount of evidence has shown that physical and psychological stress elevates plasma levels of several cytokines in animals and humans (i.e. TNF-α, IL-6, IFNγ), the physiological significance of such elevation remains to be elucidated (Yamasu et al. 1992; Nukina et al. 1998; Maes et al. 1998; Dunn et al. 1999).

One of these cytokines, tumor necrosis factor-alpha (TNF-α) is rapidly produced in the brain in response to tissue injury. This cytokine is released in its soluble form from its membrane-bound precursor by a membrane-anchored zinc metalloproteinase, identified as a disintegrin and metalloproteinase (ADAM) called TNF-α convertase (TACE/ADAM17) (Gearing et al. 1994; Black et al. 1997; Moss et al. 1997; Rosendahl et al. 1997). TNF-α exerts some of its effects through the activation of a pro-inflammatory transcription factor, nuclear factor κB (NF-κB) (rev. in Li and Karin 1999). NF-κB is held in an inactive form in the cytosol by interaction with inhibitory proteins (IκB); in this context, TNF-α leads to phosphorylation of IκB, resulting in release and translocation of NF-κB to the nucleus, where it induces transcription of target genes. Its activation is an essential requirement for the expression of the iNOS gene (Xie et al. 1994).

We have therefore decided to investigate whether TACE-mediated TNF-α release might be involved in the mechanisms by which stress leads to the expression of iNOS in rat brain. In addition to this, we investigated whether this is an excitatory amino acid (EAA) dependent process. Indeed, the neurotoxic action of glutamate and other EAA mainly through *N*-methyl-D-aspartate (NMDA) receptor have been implicated as one of the main events in the pathogenesis of stress-induced brain injury (Sapolsky et al. 1990; Moghaddam 1993; Magariños and McEwen 1995; Kim et al. 1996), a hypothesis which has been further supported by our recent work showing that MK-801 inhibits stress-induced NF-κB activation (Madrigal et al. 2001a).

MATERIALS AND METHODS

Animals

Adult male Wistar rats weighing 225–250 g were used. All experimental protocols adhered to the guidelines of the Animal Welfare Committee of the Universidad Complutense. The rats were housed individually under standard conditions of temperature and humidity and a 12 h light/dark cycle (lights on at 8 A.M.) with free access to food and water. All animals were maintained under constant conditions for four days prior to stress.

Immobilization Stress

Rats were exposed to stress between 9 A.M. and 3 P.M. in the animal homeroom. The immobilization was performed using a plastic rodent restrainer (Decapi-cone®, Braintree) that allowed for a close fit to rats. Animals were sacrificed (still in the restrainer) using sodium pentobarbital. After decapitation, brain was removed from the skull and both cortical areas were excised from the brain.

TACE Activity in Tissue Homogenates

TACE activity was monitored as the ability to cleave a 12-residue peptide spanning the Ala^{76}-Val^{77} site in pro-TNF. TACE activity was determined after sonication of the tissues (Labsonic 2000) at 4°C in 5 volumes of buffer containing 320 mM sucrose, 1 mM DL-dithiothreitol, 10 μg/ml leupeptin, 0.5 mM PMSF (phenylmethylsulphonyl fluoride), 10 μg/ml soybean trypsin inhibitor, 2 μg/ml aprotinin, 5 mM NaF, 1 mM $NaVO_4$, 10 mM $NaMoO_4$, 0.2% Nonidet and 50 mM Tris brought to pH 7.0 at 20°C with HCl. The fluorophoric peptide substrate fluorescein-SPLAQAVRSSSR-Lys-biotin (pro-peptide) was dissolved to a final concentration of 0.025 mg/ml in assay buffer containing 5 mM NaF, 1 mM $NaVO_4$, $NaMoO_4$, 100 μM $ZnCl_2$ and 50 mM Tris-HCl brought to pH 7.4 at 37°C with HCl. The homogenate was diluted in assay buffer (1:10) and allowed to incubate for 20 min at 37°C. The unhydrolyzed pro-peptide and the biotin-linked, non-fluorescent peptide product VRSSSR-Lys-biotin were removed using an avidin resin (avidin immobilized on cross-linked 6% beaded agarose). The fluorescence of the peptide product fluorescein-SPLAQA was measured in a fluorescence microplate reader (Fluoroskan FL Ascent, Labsystems). The activity of TACE was calculated from the difference be-

tween the fluorescence produced from control samples and samples containing 100 μM 1,10-phenanthroline.

TNF-α Levels

A rat TNF-α ELISA kit (R&D systems) was used for *in vitro* quantitative determination of TNF-α.

NO Synthase Activity

Cortices were snap-frozen in liquid nitrogen and stored until assayed at −80 °C. Frozen tissues were homogenized by sonication (VibraCell) in an ice-cold buffer (pH 7.4) containing Tris HCl (50 mM), sucrose (320 mM), dithiothreitol (1 mM), leupeptin (10 μg/ml), soybean trypsin inhibitor (10 μg/ml), and aprotinin (2 μg/ml) (homogenization buffer), followed by centrifugation at 10,000 g for 20 min at 4 °C. Nitric oxide synthase (NOS) activity was determined as described (Salter et al. 1991; Rees et al. 1995).

Characterization of iNOS and TACE by Western Blot

Tissues were homogenized in homogenization buffer. For determination of TACE protein, samples were deglycosylated as described by Lammich et al. (1999). 0.2% Nonidet and 100 μM 1,10-phenanthroline were included in homogenization buffer. 1,10-phenanthroline was included to avoid removal of the cytoplasmic domain after cell lysis by a metalloprotease, very likely TACE itself (Schlöndorff et al. 2000). For iNOS, the post-mitochondrial supernatant obtained after centrifugation of the homogenate at 12,000 g for 20 min at 4°C was used. The proteins present in the supernatant were loaded (10 μg) and size-separated in 10% sodium dodecyl sulphate-polyacrilamide gel electrophoresis (90 V). The proteins were blotted onto a PVDF (polyvinylidene difluoride) membrane (Millipore) and incubated with a specific polyclonal anti-TACE (Chemicon, 1:1000 dilution) or iNOS antibody (Santa Cruz Biotechnology; 1:1000 dilution). Proteins recognized by the antibody were revealed by ECL® technique following manufacturers instructions (Amersham). Autoradiographs were quantified by laser densitometry (Molecular Dynamics), and several exposure times were analyzed to ensure the linearity of the band intensities.

Preparation of Nuclear Extracts

A modified procedure based on the method of Schreiber et al. (1989) was used. Tissues (brain cortex) were homogenized with 300 μl of buffer A (10 mM HEPES, pH 7.9; 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, 0.1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml TLCK (Nα-p-tosyl-L-lysine-chloromethyl ketone), 5 mM NaF, 1 mM $NaVO_4$, 0.5 M sucrose and 10 mM Na_2MoO_4). After 15 min, Nonidet P-40 was added to reach a 0.5% concentration. The tubes were gently vortexed for 15 s and nuclei were collected by centrifugation at 8,000 g for 5 min. The pellets were resuspended in 100 μl of buffer A supplemented with 20% glycerol and 0.4 M KCl, and gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 g for 5 min, and aliquots of the supernatant were stored at -80°C. All steps of the fractionation were carried out at 4°C.

Electrophoretic Mobility Shift Assay (EMSA) for NF-κB

Oligonucleotides were synthesized in a oligonucleotide synthesizer (Pharmacia). The oligonucleotide sequence corresponding to the consensus NF-κB binding site (nucleotides -978 to -952) of the murine iNOS promoter, was 5′TGCTAGGGGGATTTTCCCTCTCTCTGT3′ (Xie et al. 1994). Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. Aliquots of 50 ng of these annealed oligonucleotides were end-labeled with Klenow enzyme (Amersham) fragment in the presence of 50 μCi of [α-^{32}P]dCTP (Amersham) and the other unlabeled dNTPs in a final volume of 50 μl. 5×10^4 dpm of the DNA probe were used for each binding assay of nuclear extracts as follows: 15 μg of protein were incubated for 15 min at 4°C with the DNA and 2 μg of poly(dI:dC), 5% glycerol, 1 mM EDTA, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM Tris-HCl, pH 7.8, in a final volume of 20 μl. The DNA-protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris borate-EDTA buffer (Díaz-Guerra et al. 1996). Supershift assays were carried out after incubation of the nuclear extract with the antibody (0.5 μg) for 1 hr at 4°C, followed by EMSA. The bands of the autoradiography were quantified by laser densitometry (Molecular Dynamics).

Pharmacological Treatments

Groups of 6–8 animals were i.p. injected with BB1101 (a TACE inhibitor; Kupatt et al. 1999; Watts et al. 1999), with MK-801 ([(+)-5-methyl-10,11-dihydroxy-5H-dibenzo (a,d) cyclohepten-5,10-imine]; dizocilpine), a specific non-competitive NMDA antagonist (Wong et al. 1988) or with PDTC (pyrrolidine dithiocarbamate, an inhibitor of the activation of NF-κB; Schreck et al. 1992). Drugs were administered at the onset of the stress.

Protein Assay

Proteins were measured using bicinchoninic acid (Hill and Straka 1988).

Chemicals and Statistical Analyses

BB1101 was a kind donation from British Biotech, the fluorescent pro-peptide substrate was synthesized by Dr. D. Andreu (Peptide Synthesis Unit, Universidad de Barcelona), MK-801 hydrogen maleate was obtained from RBI/Sigma, N-glycosidase F was from Roche and unless otherwise stated, the other chemicals were from Sigma. Results are expressed as mean ± SEM of the indicated number of experiments; statistical comparisons were made using a Newman-Keuls test and $p < .05$ was considered as statistically significant.

RESULTS

Effect of Stress on Brain TACE Activity and Protein and on Soluble TNF-α Levels and Effect of BB1101

Acute immobilization increased TACE activity in cortex as soon as 30 min after the onset of the stress (control: 5.90 ± 0.34 pmol/min per mg protein) and this increase persisted after one hour (Figure 1, Panel A). On the other hand, exposure to immobilization stress did not modify the levels of mature TACE protein as shown by immunoblotting (Figure 1, Panel A, inset). Chronic stress (6 h of immobilization during 21 consecutive days) did not modify TACE activity (102.2% $p > .05$ vs. control, non-stressed animals).

In addition, soluble TNF-α levels in brain cortical samples from 1-h–immobilization-stressed animals were higher than those found in control animals (control: 30.3 ± 0.7 pg/ml) (Figure 1, Panel B). The intraperitoneal administration of the preferred TACE inhibitor BB1101 (2 and 10 mg/kg) before the immobilization period inhibited stress-induced TNF-α release (Figure 1, Panel B).

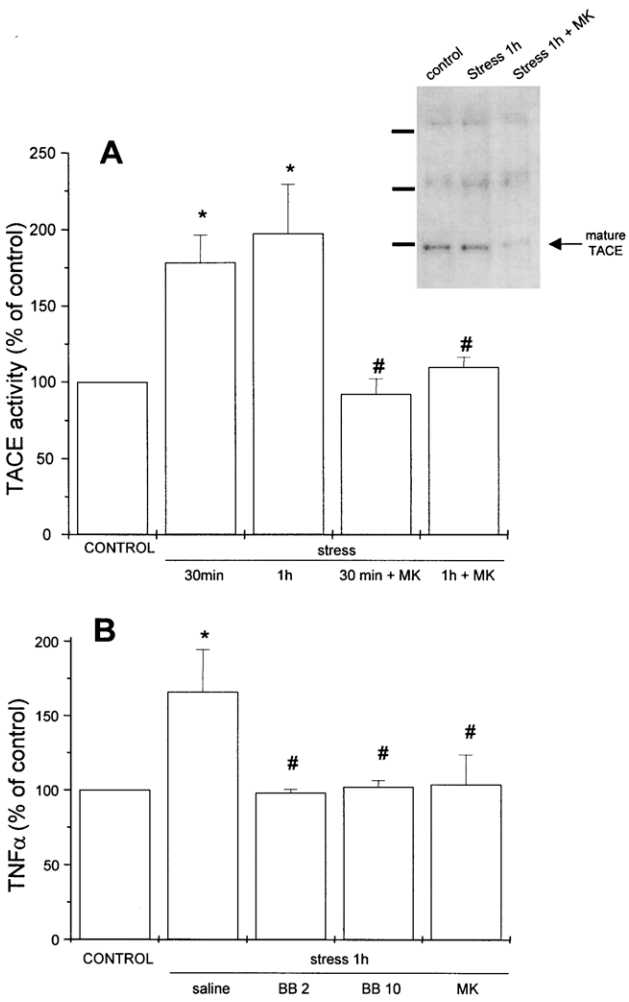

Figure 1. **(A)** TACE activity in brain cortex from control and stressed rats after 30 min–1 h of immobilization. Effect of MK-801 (MK, 0.2 mg/kg i.p.) on the stress-induced increase in TACE activity in brain cortex after 30 min–1 h. Data are mean ± SEM, n = 6, $^{*}p < 0.05$ vs. control; $p < 0.05$ vs. stress 30 min or stress 1 h, respectively (Newman-Keuls test). (Inset: detection of mature TACE protein by Western blot. Left: prestained commercial marker, from top to bottom: 209, 120 and 78 kDa). **(B)** TNF-α levels in cortical samples from rats after 1 h of immobilization injected with saline, BB1101 (2 and 10 mg/kg i.p.) or MK-801 (0.2 mg/kg i.p.). Data are mean ± SEM, n=8, * $p < .05$ vs. control; # $p < .05$ vs. saline (Newman-Keuls test).

Effect of TACE Inhibition on Stress-induced NF-κB Activation

Acute immobilization caused the translocation of NF-κB to the nucleus when measured four hours after the beginning of stress exposure (500.0 ± 10.0% of control in p50/p65 heterodimer) (Figure 2). Shorter periods of stress exposure did not cause a detectable NF-κB translocation to the nucleus (data not shown). In addition, chronic stress did not modify NF-κB translocation (114.4%, $p > .05$ vs. control, non-stressed animals).

Administration of BB1101 (2 and 10 mg/kg) inhibited stress-induced NF-κB translocation to the nucleus (47.5 ± 5.0% of inhibition from stress in p50/p65 heterodimer with 10 mg/kg of BB1101; Figure 2). As a control of pharmacological inhibition of NF-κB, samples from stressed rats injected with the NF-κB inhibitor PDTC (150 mg/kg i.p.) immediately before stress were assayed (Figure 2). The dose of PDTC was chosen for its clean inhibition of NF-κB activation, iNOS activity (Madrigal et al. 2001a) and also because it is devoid of toxicity when used in systemic administration.

Effect of TACE Inhibition on Stress-induced iNOS Expression

Acute immobilization caused the appearance of a calcium-independent NOS activity in cortex after six hours of stress (Figure 3). Calcium-dependent NOS activity re-

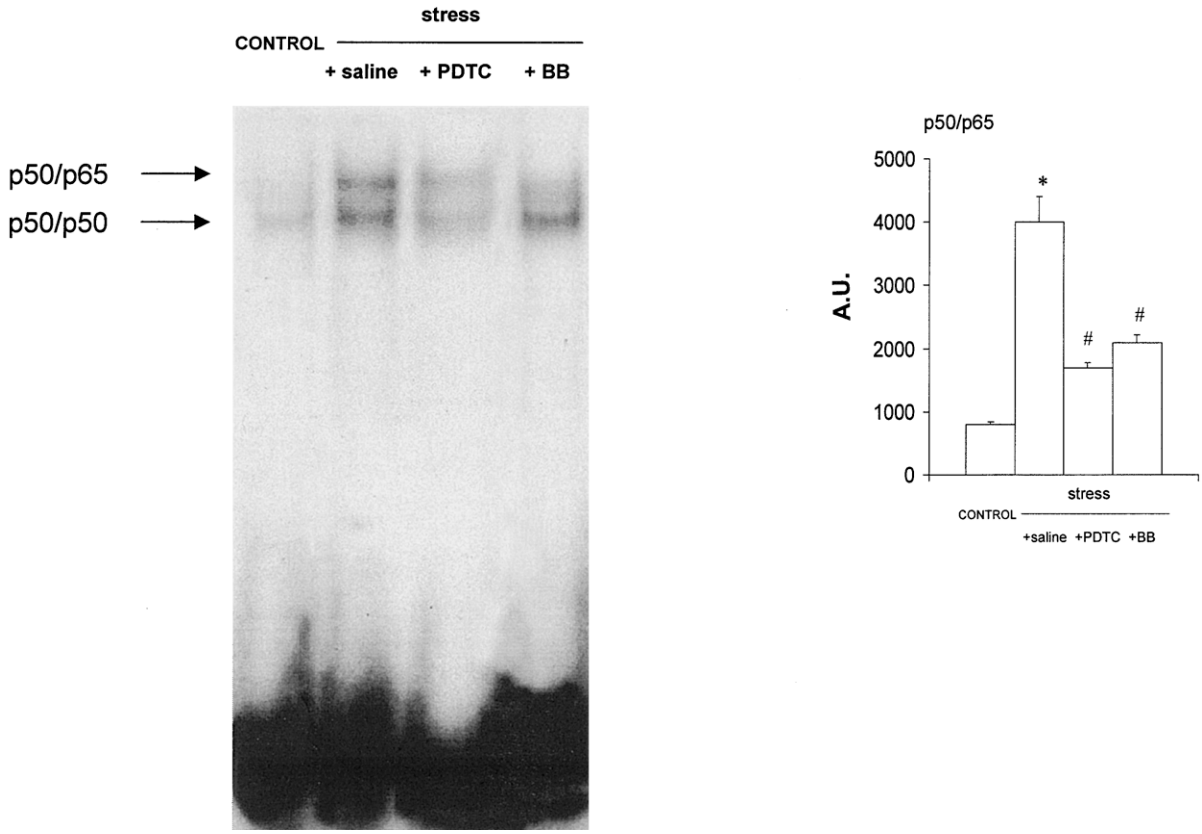

Figure 2. NF-κB translocation to the nucleus caused by stress. NF-κB translocation was measured by electrophoretic mobility shift assay (EMSA, left panel; see Materials and Methods) in control rats and four hours after the onset of stress in rats receiving saline, PDTC (150 mg/kg i.p.) or BB 1101 (10 mg/kg i.p.). Laser densitometric analysis (arbitrary units, A.U.) of the p50/p65 band (right panel). Results are representative of 3 individual experiments. $^{*}p < .05$ vs. control; $^{\#}p < .05$ vs. saline (Newman-Keuls test).

mained unchanged during the same period of time (203.8 ± 80.0 and 170.8 ± 59.0 pmol/min mg protein in control and 6 h-stressed animals, respectively). The calcium-independent activity corresponded to an iNOS protein as seen by Western blot (Figure 3, inset). This iNOS protein is still present after 21 days of immobilization (485.6% from control, $p > .05$; Olivenza et al. 2000).

Administration of BB1101 inhibited stress-induced expression of iNOS activity and protein in cortex (Figure 3) after six hours of stress. BB1101 did not modify the calcium-dependent NOS activity (BB1101, 10 mg/kg: 171.0 ± 36.0 pmol/min per mg protein, $p > .05$ vs. control).

Effect of an NMDA Antagonist on Stress-induced Increase in TACE Activity and TNF-α Levels

Administration of 0.2 mg/kg i.p. of MK-801 at the beginning of immobilization abolished stress-induced increase in TACE activity (Figure 1, Panel A) as well as TNF-α levels (Figure 3, Panel B) in brain cortex. Interestingly, mature TACE protein levels were decreased after MK-801 treatment in stressed animals (Figure 1, Panel A, inset).

DISCUSSION

We have recently demonstrated using biochemical, immunohistochemical and western blot analyses that iNOS is expressed in brain cortex after repeated exposure to immobilization stress, leading to NO_3^- and NO_2^- accumulation and oxidative damage in brain (Olivenza et al. 2000). Furthermore, we showed that aminoguanidine, a selective inhibitor of iNOS, decreases structural and functional damage caused after three weeks exposure to immobilization in rats (Olivenza et al. 2000; Madrigal et al. 2001b). Now we have found that a shorter duration of stress (immobilization for 6 h) induces the expression of iNOS in brain cortex from adult male rats, which is preceded by an increase in TACE expression, with the subsequent release of TNF-α. In addition, our data demonstrate that the resulting overproduction of TNF-α is involved in stress-induced expression of iNOS in this model *via* a NFκB-dependent mechanism. Finally, we have found that the activation of TACE is dependent on glutamate receptor activation.

Our findings showing that acute stress leads to iNOS expression pointed to the interest of the study of the

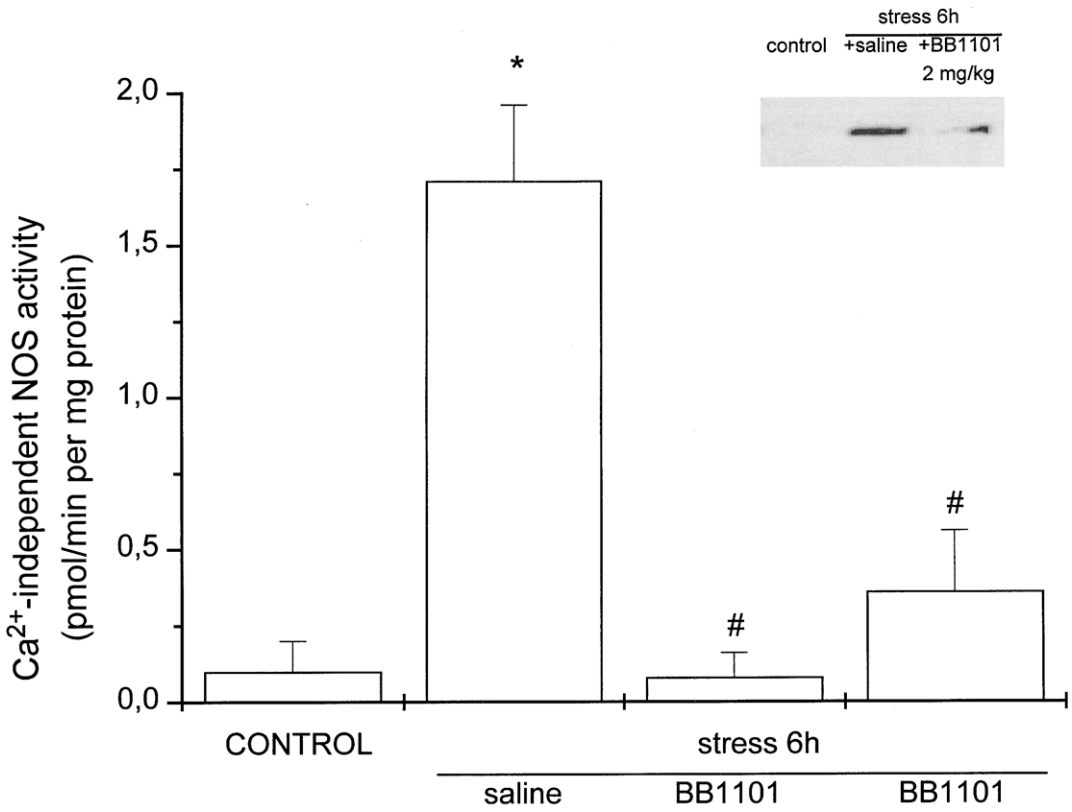

Figure 3. Calcium-independent nitric oxide synthase (NOS) activity in cortex from control and stressed rats by immobilization during 6 hours and stressed rats receiving BB-1101 (2 and 10 mg/kg i.p.). NO synthase activity was measured by monitoring the conversion of L-[U^{14}C]arginine into L-[U^{14}C]citrulline (see Materials and Methods). The data represent the mean ± SEM of 6–8 rats. $^{*}p < .05$ vs. control; $^{\#}p < .05$ vs. stress 6 h (Newman-Keuls test). Inset: detection of iNOS protein by Western blot.

mechanisms by which iNOS is stimulated, since this NOS isoenzyme mediates citotoxicity in many cell systems including brain (rev. in Moncada et al. 1991; Gross and Wolin 1995). Contrarily to the neuronal and endothelial NO synthases, iNOS is mainly regulated at the transcriptional level (Stuehr and Marletta 1987; Förstermann and Kleinert 1995) and its expression occurs after exposure to several stimuli such as cytokines (rev. in Nathan and Xie 1994). In this context, it is well known that cytokines are released in brain in animal models of stress (immobilization) (Minami et al. 1991; Shintani et al. 1995) as well as in plasma samples of humans subjected to psychological stress, anxiety states and anorexia nervosa (Ackerman et al. 1998; Holden and Pakula 1999). A corollary of these evidences is that TNF-α overproduction resulting from stress-induced TACE up-regulation could mediate the induction of iNOS. Indeed, BB1101, a preferred inhibitor of metalloproteinases with sheddase activity (Kupatt et al. 1999; Watts et al. 1999) inhibited not only stress-induced TNF-α release but also the expression of iNOS as demonstrated at the level of iNOS activity and protein, suggesting that TNF-α is involved in the expression of this NOS isoform in our model.

As regards to the mechanisms for the increase in TNF-α levels, we have found that *in vitro* TACE enzymatic activity in brain cortex is increased after immobilization stress. In this context, TACE activation seems not to require new mRNA transcription and protein synthesis in many cell types (Black et al. 1997; Watanabe et al. 1998), in agreement with our results showing that, although the catalytic activity of TACE is increased, its protein levels remain unaffected. Stress-induced increase in TACE catalytic activity is likely to be regulated by phosphorylation, as it has been reported that metalloprotease-induced protein ectodomain shedding of a large variety of cell surface proteins can be triggered by addition of phorbol esters to cells, presumably by activating PKC (Arribas et al. 1996; Hooper et al. 1997). Concerning the analysis of the expression of TACE protein, several immunopositive bands have been described to be detectable with specific anti-TACE antibodies, corresponding to glycosylated and non-glycosylated precursors and mature protein. Deglycosylation was required in this model in order to recognize an immunopositive band at approximately 80 kDa, the size corresponding to the mature form of TACE/ADAM17. We have reported recently that TACE is up-regulated by increased protein levels in the ischemic rat brain (Hurtado et al. 2001), suggesting different mechanisms of physiopathological regulation in these disorder.

On the other hand, we have recently demonstrated that NF-κB translocation to the nucleus occurs after four hours of stress and precedes iNOS expression in brain cortex, an effect that is inhibited by the NMDA antagonist MK-801 (Madrigal et al. 2001a). Interestingly, we have now shown that specific pharmacological inhibition of TACE (by BB1101) leads to an inhibition of stress-induced NF-κB translocation to the nucleus. This suggests that, in our model, TNF-α leads to iNOS expression *via* NF-κB translocation. The activation of the transcription factor NF-κB, i.e. the nuclear transport of NF-κB subunits, p50/p65, p50/p50, has been identified as an essential requirement for the expression of iNOS (Xie et al. 1994). A wide variety of noxious stimuli, bacteria and viruses, UV light, ionizing radiation, free radicals, cytokines, as well as several molecules playing key roles in neural function and development, growth factors and neurotransmitters, activate NF-κB, which in turn positively regulates the expression of genes that mediate the inflammatory response (Baldwin 1996; Baeuerle and Baichwal 1997; Mattson et al. 2000). The two bands recognized by EMSA contained p50/p65 heterodimers (upper band) and p50 homodimers (lower band). It is to note that most of the transcriptional activity is mediated through the p50/p65 complexes, whereas p50 homodimers exert an inhibitory action (Baldwin 1996). Indeed, basal levels of p50 homodimers are detected by EMSA in various tissue extracts. In this vein, the accumulation of p50/p50 dimers elicited after BB1101 treatment suggests the existence of a specific pathway of impairment of NF-κB activity the meaning of which in the context of stress deserves further attention. It also remains to be studied whether this is linked to the expression of other proinflammatory mediators.

Furthermore, after the initial observation that extracellular concentrations of glutamate and aspartate are increased in the rat brain during the initial stages of stress, as soon as 20 min after the onset of immobilization (Moghaddam 1993), various studies have shown that the neurotoxic actions of these excitatory amino acids mainly through NMDA receptor have been implicated in the pathogenesis of stress-induced brain injury (Magariños and McEwen 1995), as occurs in a wide range of other neurological disorders (Olney 1990; Zorumski and Olney 1993). We demonstrate here that NMDA receptor activation accounts, at least in part, for the increase in TACE activity and TNF-α production in this particular model. This finding may explain the inhibition of iNOS expression that we have observed after MK-801 administration in this model (Madrigal et al. 2001a) and in an *in vitro* model of brain ischemia (Cárdenas et al. 2000).

This, together with our findings showing that MK-801 inhibits stress-induced increase in TACE activity, suggest that all these changes seem to occur in a time-dependent manner: early after the onset of the stress (30–60 min), the overactivation of the NMDA receptor by glutamate may cause the activation of TACE, leading to release of soluble TNF-α (1 h), which, probably *via* phosphorylation of IκB (Li and Karin 1999), results in translocation of NF-κB to the nucleus after four hours and, finally, to iNOS expression after six hours. A striking observation which merits further study concerns the decrease in the levels of mature TACE protein in stressed animals after the administration of MK-801. Although our findings showing that MK-801 does not affect the translocation of the p50/p50 homodimer (Madrigal et al. 2001a) might suggest a regulation at this level, this is at least partly excluded by the fact that the pharmacological inhibition of TACE leads to a similar effect on NF-κB activation.

Interestingly, although the pathway glutamate-TACE-iNOS accounts for iNOS expression after acute stress, we have found that both TACE activity and NF-κB translocation have decreased to control levels after chronic stress, a situation in which iNOS is still present. Now it remains to study whether chronic stress down-regulates these systems with time or alternatively, whether other mechanisms are switched on which are able to induce iNOS expression at these times.

In addition to its role on iNOS expression, the increase in soluble TNF-α after stress may be involved in other tasks. For example, TNF-α has been reported to provoke an anxiogenic response in the elevated plus maze test of anxiety in rodents (Connor et al. 1998). In addition, whether our findings are relevant to trigger the cytokine cascade remains to be established, but it is very well known that among the proinflammatory cytokines, TNF-α exerts a special role since its activation switches on the process of synthesis of others such as IL1α, IL1β, IL6 and TNF-α itself in brain (Sharif et al. 1993).

To our knowledge, this is the first report showing a functional implication of TACE in the expression of iNOS caused by acute immobilization stress, a role to be added to a remarkably diverse set of tasks which has been described for metalloprotease-disintegrins (for rev., see Wolfsberg and White 1996; Blobel 1997; Black and White 1998).

In summary, these data show that the activation of TACE by stress is involved in the expression of iNOS in rat brain cortex *via* the release of TNF-α. These findings could provide novel therapeutic targets in a wide range of neuropathologic disorders, which include several degenerative diseases where TNF-α as well as NO and products of its further oxidation are implicated.

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REFERENCES

Ackerman KD, Martino M, Heyman R, Moyna NM, Rabin BS (1998): Stressor-induced alteration of cytokine production in multiple sclerosis patients and controls. Psychosom Med 60:484–491

Arribas J, Coodly L, Vollmer P, Kishimoto TK, Rose-John S, Massague J (1996): Diverse cell surface protein ectodomains are shed by a system sensitive to metalloprotease inhibitors. J Biol Chem 10:11376–11382

Baeuerle PA, Baichwall VR (1997): NF-kappa B as a frequent target for immunosuppressive and anti-inflammatory molecules. Adv Immunol 65:111–137

Baldwin Jr AS (1996): The NF-kappa B and I kappa-B proteins: new discoveries and insights. Annu Rev Immunol 14:649–683

Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Scooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP (1997): A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. Nature 385:729–733

Black RA, White JM (1998): ADAMs: focus on the protease domain. Curr Opin Cell Biol 10:654–659

Blobel CP (1997): Metalloprotease-disintegrins: Links to cell adhesion and cleavage of TNFα and Notch. Cell 90:589–592

Bremner JD, Southwick SM, Charney DS (1991): Animal models for the neurobiology of trauma. PTSD Res Quart 2:1–7

Cárdenas A, Moro MA, Hurtado O, Leza JC, Lorenzo P, Castrillo A, Bodelón OG, Boscá L, Lizasoain I (2000): Implication of glutamate in the expression of inducible nitric oxide synthase after oxygen and glucose deprivation in rat forebrain slices. J Neurochem 74:2042–2048

Connor TJ, Song C, Leonard BE, Merali Z, Anisman H (1998): An assessment of the effects of central interleukin-1beta, -2, -6, and tumor necrosis factor-alpha administration on some behavioural, neurochemical, endocrine and immune parameters in the rat. Neuroscience 84:923–933

Díaz-Guerra MJM, Velasco M, Martín-Sanz P, Boscá L (1996): Evidence for common mechanisms in the transcriptional control of type II nitric oxide synthase in isolated hepatocytes. Requirement of NF-κB activation after stimulation with bacterial cell wall products and phorbol esters. J Biol Chem 271:30114–30120

Dunn AJ, Wang J, Ando T (1999): Effects of cytokines on cerebral neurotransmission. Comparison with the effects of stress. Adv Exp Med Biol 461:117–127

Förstermann U, Kleinert H (1995): Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Arch Pharmacol 352:351–364

Gearing AJH, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, Drummond AH, Galloway WA, Gilbert R, Gordon JL, Leber TM, Mangan M, Miller K, Nayee P, Owen K, Patel S, Thomas W, Wells G, Wood LM, Woolley K (1994): Processing of tumour necrosis factor-α precursor by metalloproteinases. Nature 370:555–557

Gross SS, Wolin MS (1995): Nitric oxide: pathophysiological mechanisms. Annu Rev Physiol 57:737–769

Hill HD, Straka JG (1988): Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. Anal Biochem 170:203–208

Holden RJ, Pakula IS (1999): Tumor necrosis factor-alpha: is there a continuum of liability between stress, anxiety states and anorexia nervosa? Med Hypoth 52:155–162

Hooper NM, Karran EH, Turner AJ (1997): Membrane protein secretases. Biochem J 321:265–279

Hurtado O, Cárdenas A, Lizasoain I, Boscá L, Leza JC, Lorenzo P, Moro MA (2001): Up-regulation of TNF-α convertase (TACE/ADAM17) after oxygen-glucose deprivation in rat forebrain slices. Neuropharmacology 40:1094–1102

Kim JJ, Foy MR, Thompson RF (1996): Behavioral stress modifies hippocampal plasticity through N-methyl-D-aspartate receptor activation. Proc Natl Acad Sci USA 93:4750–4753

Kim JJ, Yoon KS (1998): Stress: metaplastic effects in the hippocampus. Trends Neurosci 21:505–509

Kupatt C, Habazettl H, Goedecke A, Wolf DA, Zahler S, Boekstegers P, Kelly RA, Becker BF (1999): Tumor necrosis factor-α contributes to ischemia- and reperfusion-induced endothelial activation in isolated hearts. Circ Res 84:392–400

Lammich S, Kojro E, Postina R, Gilbert S, Pfeiffer R, Jasionowski M, Haass C, Fahrenholz F (1999): Constitutive and regulated α-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proc Natl Acad Sci USA 96:3922–3927

Leza JC, Salas E, Sawicki G, Russell JC, Radomski MW (1998): The effect of stress on homeostasis in JCR:LA-cp rats: the role of nitric oxide. J Pharmacol Exp Ther 286:1397–1403

Li N, Karin M (1999): Is NFκ-B the sensor of oxidative stress? FASEB J 13:1137–1143

Madrigal JLM, Moro MA, Lizasoain I, Lorenzo P, Castrillo A, Boscá L, Leza JC (2001a): Inducible nitric oxide synthase expression in brain cortex after acute restraint stress is regulated by nuclear factor κB - mediated mechanisms. J Neurochem 76:532–538

Madrigal JLM, Olivenza R, Moro MA, Lizasoain I, Lorenzo P, Leza JC (2001b): Glutathione depletion, lipid peroxidation and mitochondrial disfunction are induced by chronic stress in rat brain. Neuropsychopharmacology 24:420–429

Maes M, Song C, Lin A, De Jongh R, Van Gastel A, Kenis G, Bosmans E, De Meester I, Benoy I, Neels H, Demedts P, Janca A, Scharpe S, Smith RS (1998): The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. Cytokine 10:313–318

Magariños AM, McEwen BS (1995): Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors. Neuroscience 69:89–98

Mattson MP, Culmsee C, Yu Z, Camandola S (2000): Roles of nuclear factor -κB in neuronal survival and plasticity. J Neurochem 74:443–456

Minami M, Guraishi Y, Yamaguchi T, Nakai S, Hirai Y, Satoh M (1991): Immobilisation stress induces interleukin-1β mRNA in the rat hypothalamus. Neurosci Lett 123:254–256

Moghaddam B (1993): Stress preferentially increases extraneuronal levels of excitatory amino acids in the prefrontal cortex: comparison to hippocampus and basal ganglia. J Neurochem 60:1650–1657

Moncada S, Palmer RMJ, Higgs EA (1991): Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev 43:109–142

Moss ML, Jin S-LC, Milla ME, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su J-L, Warner J, Willard D, Becherer JD (1997): Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-α. Nature 385:733–736

Nathan C, Xie Q-W (1994): Nitric oxide synthases: roles, tolls and controls. Cell 78:915–918

Nukina H, Sudo N, Komaki G, Yu X, Mine K, Kubo C (1998) The restraint stress-induced elevation in plasma interleukin-6 negatively regulates the plasma TNF-alpha level. Neuroimmunomodulation 5:323–327

Olney JW (1990): Excitotoxic amino acids and neuropsychiatric disorders. Annu. Rev Pharmacol Toxicol 30:47–71

Olivenza R, Moro MA, Lizasoain L, Lorenzo P, Fernández AP, Rodrigo J, Boscá L, Leza JC (2000): Chronic stress induces the expression of inducible nitric oxide synthase in rat brain cortex. J Neurochem 74:785–791

Rees DD, Cunha FQ, Assreuy J, Herman AG, Moncada S (1995): Sequential induction of nitric oxide synthase by Corynebacterium parvum in different organs of the mouse. Br J Pharmacol 114:689–693

Rosendahl MS, Ko SC, Long DL, Brewer MT, Rosenzweig B, Hedl E, Anderson L, Pyle SM, Moreland J, Meyers MA, Kohno T, Lyons D, Lichenstein HS (1997): Identification and characterization of a pro-tumor necrosis factor-α-processing enzyme from the ADAM family of zinc metalloproteases. J Biol Chem 272:24588–24593

Salter M, Knowles RG, Moncada S (1991): Widespread tissue distribution, species distribution and changes in activity of Ca2+-dependent and Ca2+-independent nitric oxide synthases. FEBS Lett 291:145–149

Sapolsky R (1996): Why stress is bad for your brain. Science 273:749–750

Sapolsky R, Uno H, Robert C, Finch C (1990): Hippocampal damage associated with prolonged glucocorticoid exposure in primates. J Neurosci 10:2897–2904

Schlöndorff J, Becherer JD, Blobel CP (2000): Intracellular maturation and localization of the tumour necrosis factor alpha convertase. Biochem J 347:131–138

Schreck R, Meier B, Männel DN, Dröge W, Baeuerle PA (1992): Dithiocarbamates as potent inhibitors of nuclear factor kappa B activation in intact cells. J Exp Med 175:1181–1194

Schreiber E, Matthias P, Müller MM, Schaffner W (1989): Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acid Res 17:6419

Sharif SF, Hariri RJ, Chang VA, Barie PS, Wang RS, Ghajar JB (1993): Human astrocyte production of tumor necrosis factor-alpha, interleukin-1 beta and interleukin-6 following exposure to lipopolysaccharide endotoxin. Neurol Res 15:109–112

Sheline YI, Wang PW, Gado MH, Csernansky JG (1996): Hippocampal atrophy in recurrent major depression. Proc Natl Acad Sci USA 93:3908–3913

Shintani F, Nakaki T, Kanba S, Sato K, Yagi G, Shiozawa M, Aiso S, Kato R, Asai M (1995): Involvement of interleukin-1 in immobilization stress-induced increase in plasma adrenocorticotropic hormone and in release of hypothalamic monoamines in the rat. J Neurosci 15: 1961–1970

Stuehr DJ, Marletta MA (1987): Induction of nitrite/nitrate synthesis in murine macrophages by BCG infection, lymphokines, or interferon γ. J Immunol 139:518–525

Watanabe N, Nakada K, Kobayashi Y (1998): Processing and release of tumor necrosis factor α. Eur J Biochem 253: 576–582

Watts AD, Hunt NH, Madigan MC, Chaudhri G (1999): Soluble TNF-alpha receptors bind and neutralize over-expressed transmembrane TNF-alpha on macrophages, but do not inhibit its processing. J Leukoc Biol 66:1005–1013

Wolfsberg TG, White JM (1996): ADAMs in fertilization and development. Dev Biol 180:389–401

Wong EHF, Knight AR, Woodruff GN (1988): [^{3}H]MK-801 labels a site on the N-methyl-D-aspartate receptor channel complex in rat brain membranes. J Neurochem 50: 274–281

Xie Q-W, Kashiwabara Y, Nathan C (1994): Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. J Biol Chem 269:4705–4708

Yamasu K, Shimada Y, Sakaizumi M, Soma G, Mizuno D (1992): Activation of the systemic production of tumor necrosis factor after exposure to acute stress. Eur Cytokine Netw 3:391–398

Zorumski CF, Olney JW (1993): Excitotoxic neuronal damage and neuropsychiatric disorders. Pharmacol Ther 59: 145–162