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Characterization of the pro-inflammatory signaling induced by protein acetylation in microglia

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Abstract

Protein acetylation regulates the extent of inflammatory responses and disturbances in protein acetylation have been proposed to play an important role in inflammatory and neurodegenerative diseases. We have recently observed that histone deacetylase inhibitors, such as trichostatin A (TSA) and SAHA, strongly potentiate the LPS induced inflammatory response in several rat and mouse inflammatory models. Our aim here was to characterise pro-inflammatory signaling mediated via increased protein acetylation and protein phosphorylation in microglial N9 cells. First we observed that TSA induced pro-inflammatory response was independent of the different Toll-like receptors activated, since LPS, flagellin and unmethylated CpG oligonucleotides, equally potentiated IL-6 secretion from N9 microglia. Next we compared the protein acetylation induced potentiation to that induced by okadaic acid, a well-known inducer of pro-inflammatory responses. The time scale of the IL-6 responses showed that the effects of okadaic acid were clearly early-response effects appearing as soon as 6 h after exposure, whereas TSA evoked a significant inhibition in IL-6 secretion up to 12 h but after that it induced an exponential increase in cytokine and nitric oxide production up to 24 h. It seems that okadaic acid induces an early moderate response and TSA a late but exponential potentiation of microglial inflammatory responses. The pro-inflammatory responses of TSA and okadaic acid were both dependent on NF-κB signaling but independent on the DNA-binding capacity of nuclear NF-κB complexes. Interestingly, we observed that the transactivation of the NF-κB-Luc reporter gene was clearly activated during TSA induced pro-inflammatory potentiation. Our studies imply that the potentiation of the inflammatory response by increased acetylation is due to the enhancement of transactivation of NF-κB driven inflammatory genes. Our studies on signaling pathways revealed that PI3K inhibitors LY294002 and Wortmannin blocked the TSA induced pro-inflammatory response but surprisingly did not affect the okadaic acid induced response. Furthermore, LY294002 did not inhibit DNA-binding activity of NF-κB but still inhibited NF-κB-Luc reporter gene transactivation. These results indicate that PI 3-kinase regulates the transactivation efficiency of NF-κB-dependent transcription rather than transduction of NF-κB signaling.

Keywords: Inflammation; Neurodegeneration; NF-κB; HDAC; LPS; N9

1. Introduction

Disturbances in the regulation of protein acetylation have been proposed to play an important role in the pathogenesis of several diseases, such as cancer (Marks et al., 2004; Hess-Stumpp, 2005), diabetes (Gray and de Meyts, 2005), neurodegenerative disorders (Mattson, 2003; Langley et al., 2005), as well as being involved in several inflammatory diseases (Chung et al., 2003; Rahman et al., 2004; Adcock et al., 2005). Treatment with histone deacetylase inhibitors have shown promising therapeutic results in some nervous system disorders, such as gliomas (Eyupoglu et al., 2005), poly-glutamine diseases (Taylor and Fischbeck, 2002; Hockly et al., 2003), and several other neurodegenerative diseases (see Langley et al., 2005).

Abbreviations: CAPE, caffeic acid phenethyl ester; Dexa, dexamethasone; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; HDAC, histone deacetylase; IKK2, I kappa B kinase 2 (IKK beta); IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; LPS, lipopoly-saccharide; Luc, Luciferase; LY, LY294002; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; OD, optical density; OKA, okadaic acid; PBS, phosphate buffered saline; PI3K, phosphoinositol 3-kinase; PMA, phorbol 12-myristate acetate; PP, protein phosphatase; NO, nitric oxide; SAHA, suberoylanilide hydroxamic acid; SLTA, lipoteichoid acid; TLR, Toll-like receptor; TSA, trichostatin A

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Inflammation is involved in several neurodegenerative diseases (Gonzalez-Scarano and Baltuch, 1999; Minghetti, 2005). Recent publications have highlighted how protein acetylation prominently modulates the inflammatory responses (Rahman et al., 2004; Barnes et al., 2005; Ito et al., 2005). We have recently observed that histone deacetylase inhibitors, such as trichostatin A and SAHA, strongly potentiate the LPS-induced inflammatory response in several rat and mouse inflammatory models in cell and slice cultures (Suuronen et al., 2003). The inflammatory responses are also modified by MAPK signaling (Shanley et al., 2001; Avdi et al., 2002). Okadaic acid, an inhibitor of protein phosphatases PP2A and PP1, increases protein phosphorylation and simultaneously potentiates the inflammatory responses (Tebo and Hamilton, 1994; Shanley et al., 2001).

In the present study, we compared the potentiation of inflammatory responses induced via the ''hyperacetylation'' or ''hyperphosphorylation'', i.e. either TSA induced or okadaic acid induced pro-inflammatory responses. Our results show that there are diverse potentiation mechanisms behind the pro-inflammatory responses mediated via acetylation and phosphorylation signaling pathways. The okadaic acid induced pro-inflammatory reaction was shown to be an early-response event whereas the TSA induced response was clearly a late event. However, both responses could be inhibited by dexamethasone and the NF-κB inhibitor. Surprisingly, the PI3K inhibitor, LY294002, blocked only the TSA induced pro-inflammatory response but did not affect the okadaic acid induced response. Our results also indicate that TSA potentiates the LPS-induced inflammatory response in N9 microglial cells by increasing the transactivation efficiency of NF-κB complexes.

2. Materials and methods

2.1. Chemicals

Lipopolysaccharide (LPS) used in all experiments was from *E. coli* 055:B5 lyophilized powder (L 6529 from Sigma). To compare the LPS purity effect, the Ultra Pure LPS purified from *E. coli* 0111:B4 strain (InvivoGen, San Diego, USA) was used. Pam3CSK4 synthetic lipoprotein, SLTA (lipoteichoid acid from *Staphylococcus aureus*), zymosan, flagellin and ODN1826 were from InvivoGen (San Diego, USA). Okadaic acid, LY294002, Wortmannin, H-7 dihydrochloride, H-89 dihydrochloride, herbimycin A, JAK3 inhibitor II, dexamethasone, IKK-2 inhibitor IV and protein kinase Cζ pseudosubstrate inhibitor (myristoylated) were from Calbiochem (Merck Biosciences, Nottingham, U.K.). Trichostatin A and MTT were purchased from Sigma. Helenalin was from BIOMOL Research Labs (Plymouth Meeting, USA) and CAPE and PMA from Sigma. Double-stranded consensus and mutated oligonucleotides for AP-1 and NF-κB binding sites used in the EMSA assays were from Santa Cruz Biotechnology (Santa Cruz, USA).

2.2. Murine N9 microglia

The murine N9 microglial cell line was established by Corradin et al. (1993). We have observed earlier that N9 microglial cells respond similarly as rat primary hippocampal microglia to histone deacetylase inhibitors (Suuronen et al., 2003). The mouse N9 microglial cell line was a kind gift from Dr. Paola Ricciardi-Castagnoli (University of Milano-Bicocca, Milan, Italy). The culture conditions for N9 microglial cells have been described earlier in detail (Suuronen et al., 2003). N9 microglial cells were plated to 12-well plates (Nunc A/S, Roskilde, Denmark) at a density of 2×10^5 cells/well for 24 h prior to drug exposures.

2.3. Rat hippocampal slice cultures

Organotypic slice cultures from rat hippocampus were established using the modified interface culture technique as described by Stoppini et al. (1991). Postnatal day 7 (P7) Wistar rat pups were decapitated, the brains rapidly dissected and placed in a petri dish in ice cold PBS (Gibco). The hippocampi of both sides were isolated and cut into 400 μm transversal slices using a McIlwain tissue chopper. The slices were then carefully separated and transferred onto porous membrane inserts (one slice per insert) of 12-well culture plates (Transwell, Costar). Half a millilitre of culture medium (Neurobasal medium with B27-supplement (Gibco), 1 mM glutamine, 100 U ml^{-1} penicillin and 100 μg/ml streptomycin) was added to the lower chamber of each well. For the first culture day, however, inactivated FBS (10%, final conc.) was added to the culture medium. The culture plates were placed in a 37 °C incubator at 5% CO_2. After 4 days *in vitro* culture, the fresh medium without B27-supplement was changed before exposing the hippocampal slices to the treatments for 24 h.

2.4. EMSA assays

EMSA assays were performed as described earlier (Helenius et al., 1996). Nuclear proteins were isolated using the modified protocol of Dignam et al. (1983). About 5 μg of nuclear protein was used in protein-DNA-binding assays. Double-stranded consensus and mutated oligonucleotides for AP-1 and NF-κB binding sites were labelled with T4 polynucleotide kinase (Promega, Madison, USA). Unspecific binding was blocked by adding 2 μg of poly(dI-dC):poly(dI-dC) (Roche Applied Science, Basel, Switzerland) into the 20 μl assay volume (Helenius et al., 1996). Protein-bound and free oligonucleotide probes were separated in a native 4% polyacrylamide gel. Radioactive bands were visualized with Storm 860 PhosphorImager (Molecular Dynamics, USA) and the pixel volumes of specific bands were calculated with ImageQuaNT 4.2a software (Molecular Dynamics).

2.5. Transactivation assays with pNFκB-Luc reporter vector

N9 microglial cells were transfected with pTAL-Luc and pNFκB-Luc Vectors from Clontech Lab (BD Biosciences, Palo Alto, USA). pNFκB-Luc is a reporter vector designed for monitoring the activation of the NF-κB signaling pathway. The pTAL-Luc vector is a negative control for determination of the background. N9 cells were transfected using FuGENE™ 6 Transfection Reagent and the proposed protocol (Roche, Penzberg, Germany). In brief, N9 cells were transfected with pTAL-Luc and pNFκB-Luc vectors (0.6 μg/ml) for 12 h in culture medium after which LPS and drugs were added for 24 h. Luciferase activity of the cells was assayed with the Luciferase kit from Promega.

2.6. ELISA, LDH and NO assays

The secretion of IL-6 was measured by ELISA using OptEIA™ kit obtained from Pharmingen (BD Biosciences, San Diego, USA). LDH leakage from the cells to medium was measured with the cytotoxicity kit obtained from Promega. The nitrite concentration in the medium was assayed by the Griess reaction, i.e. into 100 μl of sample, an equal amount of the Griess reagent (1:1 of 0.1% naphthylethylene diamide in H_2O and 1% sulfanilamide in 5% concentrated H_2PO_4) was added and the OD was measured at 550 nm using an ELISA microplate reader after 10 min incubation. MTT viability assay was performed as described recently by Kerokoski et al. (2001).

2.7. Exposure of N9 cells to LPS and inflammatory modulators

All inflammatory models were optimized before the experiments with respect to the exposure times and concentrations of LPS, TSA and signaling inhibitors. Toxicity was tested. In all experiments, LPS and histone deacetylase and signaling inhibitors were added at the same time. Incubation time was 24 h or specified in figures. Experiments were performed using 4–6 parallel samples and repeated twice or more.

2.8. Statistical analysis

All values were expressed as means ± S.D. The difference between control and treated groups was analyzed using Mann–Whitney *U*-test.

3. Results

3.1. TSA induced pro-inflammatory response is independent on different Toll-like receptors activated

LPS activates the inflammatory signaling through the Toll-like receptors (Kirschning and Bauer, 2001). First we analyzed whether TSA could enhance the inflammatory response mediated by different Toll-like receptors. It is known that commercially available LPS preparations contain other stimulating compounds, such as flagellin as impurities. We treated N9 microglia with our ordinary LPS (Sigma) and Ultra-pure LPS (InvivoGen). Fig. 1 shows that ordinary LPS induced a higher IL-6 cytokine response than Ultra-pure LPS but the potentiation of the response with TSA was very similar, approx. four-fold. LPS activates both TLR2 and TLR4 (Kirschning and Bauer, 2001). Treatment of N9 microglia with Pam3CSK4 lipoprotein SLTA and zymosan, specific TLR2 ligands, showed no responses indicating that N9 cells do not contain TLR2 receptors and that the effect of LPS, as well the potentiation by TSA, is mediated by TLR4 receptors and down-stream signaling pathways. Flagellin, a specific ligand for TLR5, and ODN1826, a specific ligand for TLR9, also increased the secretion of IL-6 and this effect was strongly potentiated by TSA treatment (Fig. 1). None of the treatments caused LDH leakage (data not shown). Toll-like receptors have pleiotrophic functions and have different pathways in signaling (Kaisho and Akira, 2004). Our results suggest that protein acetylation does not regulate inflammatory responses at the Toll-like receptor level but affects the down-stream signaling.

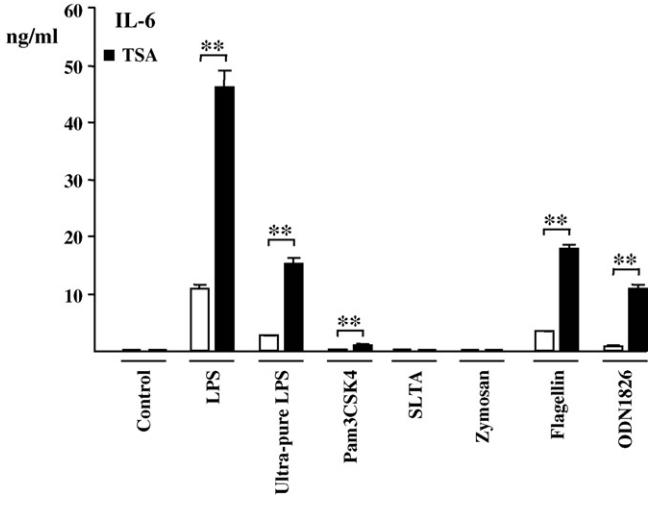

Fig. 1. Effects of the ligands for different Toll-like receptors and TSA treatment on the secretion of IL-6 in N9 microglia. LPS activates both TLR2 and TLR4 receptors. Pam3CSK4, SLTA and zymosan are specific ligands for TLR2. Flagellin is a specific ligand for TLR5 and ODN1826 for TLR9. Final concentrations of ligands: 5 μg ml^{-1} for LPS, Ultra-pure LPS and SLTA, 0.3 μg ml^{-1} for Pam3CSK4, 10 μg ml^{-1} for zymosan, 0.1 μg ml^{-1} for flagellin and 1.0 μg ml^{-1} for ODN1826. TSA concentration was 20 nM. Drug treatment was for 24 h. Values are means ± S.D. $^{**}p < 0.01$ (for the potentiation).

3.2. TSA induced pro-inflammatory response is a late event and differs from that of okadaic acid induced early response

Okadaic acid inhibits protein phosphatases PP2A and PP1 and enhances the protein phosphorylation status of cells. Okadaic acid potentiates inflammatory reactions via c-Jun N-terminal kinase (JNK) (Shanley et al., 2001). Next we compared the LPS-induced basic inflammatory reactions with each other and with the potentiated responses induced either by TSA (''acetylation response'') or okadaic acid (''phosphorylation response''). Interestingly, TSA and okadaic acid pro-inflammatory responses clearly differ from each other. Fig. 2 shows that okadaic acid potentiated LPS-induced IL-6 secretion already at 6 h but TSA caused a prominent down-regulation in IL-6 secretion as compared to LPS-only treatment. Down-regulation of LPS-induced IL-6 secretion by TSA exposure was still present after 12 h but after that time point there was a major potentiation of IL-6 secretion up to 24 h (Fig. 2). Note the different scales for the 12 and 24 h values in Fig. 2. Nitric oxide (NO), another parameter for inflammatory response, showed a slight potentiation by TSA after 24 h but a prominent inhibition by okadaic acid treatment together with

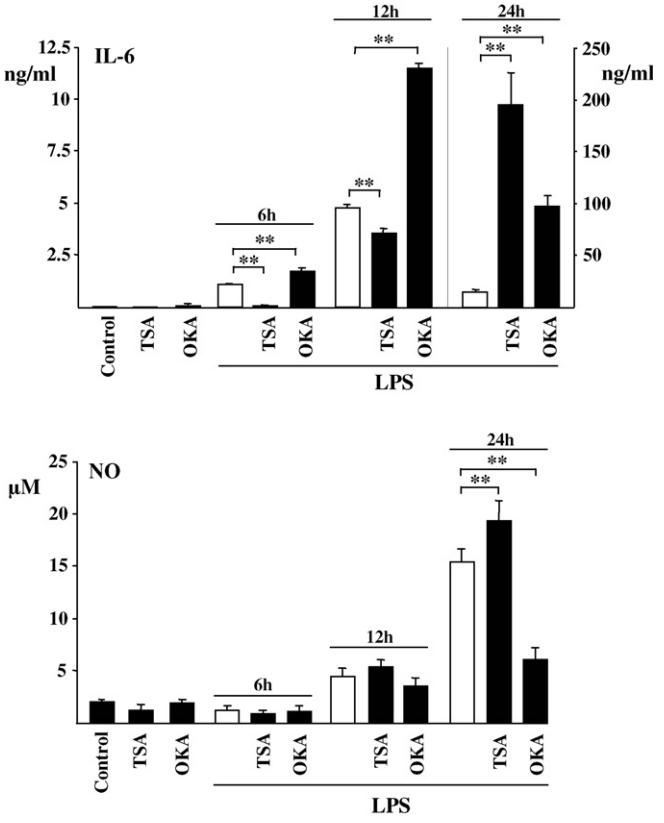

Fig. 2. Time scale of IL-6 and NO secretion activated by LPS and potentiated by okadaic acid (OKA) or TSA in N9 microglia. Final concentrations were 20 nM for TSA, 40 nM for okadaic acid and 5 μg ml^{-1} for LPS. Values are means ± S.D. $^{**}p < 0.01$ (for the potentiation/inhibition).

LPS exposure (Fig. 2). Furthermore, the simultaneous treatments with TSA and okadaic acid induced an additive response in IL-6 secretion (data not shown). These observations clearly show that there are different signaling mechanisms behind the pro-inflammatory response, i.e. ‘‘hyperacetylation’’ and ‘‘hyperphosphorylation’’.

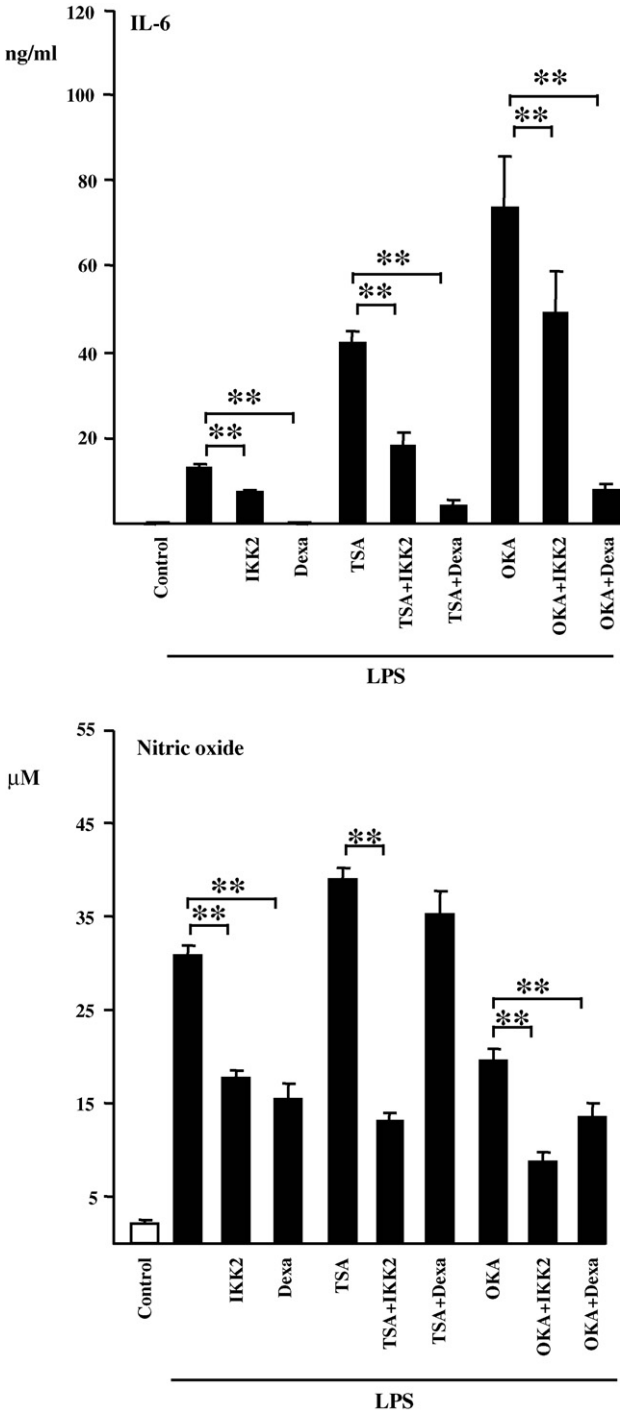

Fig. 3. Effects of IKK2 inhibitor and dexamethasone on IL-6 and NO secretion induced by LPS and potentiated by TSA or okadaic acid (OKA) in N9 microglia. Final concentrations were 0.5 μM for IKK-2 inhibitor IV and 0.5 μM for dexamethasone. LPS, OKA and TSA concentrations were as in Fig. 2. Drug treatment was for 24 h. Values are means ± S.D. $^{**}p < 0.01$ (for the inhibition).

3.3. TSA and okadaic acid induced pro-inflammatory responses are dependent on NF-κB signaling

We have observed earlier that TSA-induced potentiation of the LPS activated inflammatory response is blocked by CAPE and helenalin in N9 microglia (Suuronen et al., 2003). We have also observed that helenalin blocks the pro-inflammatory signaling induced by okadaic acid (data not shown). Helenalin is a sesquiterpene lactone which selectively alkylates the p65 subunit of NF-κB and inhibits DNA-binding of the NF-κB complex (Lyss et al., 1998). We have earlier verified that helenalin inhibits the DNA-binding of NF-κB complex and LPS-induced inflammatory response in N9 microglia (Suuronen et al., 2003). Here we extended these experiments and studied whether the TSA-induced potentiation was mediated by IkappaB kinase 2 (IKK-2), a major inflammatory IKK kinase (Hawiger et al., 1999). We used IKK-2 inhibitor IV, a specific inhibitor of IKK-2 (Podolin et al., 2005) to inhibit the IKK-2 mediated, proinflammatory NF-κB signaling pathway. Fig. 3 shows that IKK-2 inhibitor potently inhibited the un-potentiated LPS response as well as the potentiated IL-6 responses, i.e. those induced with either TSA or okadaic acid. IKK-2 inhibitor also inhibited the NO secretion in all treatments (Fig. 3). These results, together with our earlier studies (Suuronen et al., 2003), suggest that histone deacetylase inhibitors regulate the inflammatory response down-stream from the IKK signalosome at the transactivation level of the inflammatory genes.

Glucocorticoids inhibit NF-κB signaling and induce an anti-inflammatory response (Smoak and Cidlowski, 2004). The mechanism of the repression of NF-κB signaling is still mostly unknown. Our results show that dexamethasone totally blocked both the LPS-induced and the TSA-potentiated responses not only in N9 microglia (Fig. 3) but also in hippocampal slice cultures (Fig. 4). Fig. 3 shows that the effects of dexamethasone were more effective than those of IKK-2 inhibitor against both TSA and okadaic acid induced responses in IL-6 secretion but not in preventing nitric oxide secretion. Fig. 4 shows that the inhibitory effect of dexamethasone was more potent against IL-6 secretion than against nitric oxide production also in hippocampal slices. Dexamethasone also inhibited the okadaic acid-induced secretion of IL-6 and NO from hippocampal slices (data not shown). Our results indicate that dexamethasone inhibits the NF-κB mediated LPS-induced inflammatory responses but the extent of inhibition depends on individual regulation of the genes.

3.4. LPS-induced DNA-binding activity of nuclear NF-κB is not enhanced by TSA or okadaic acid treatments

The activation of NF-κB signaling in cytosol translocates NF-κB transcription factors to the nuclei (Baeuerle and Henkel, 1994). The EMSA assay for the DNA-binding activity of NF-κB complex in nuclear extract has generally been used to measure the activation level of NF-κB signaling and translocation of NF-κB components to nuclei. Fig. 5 shows that LPS exposure induced a prominent upregulation in the

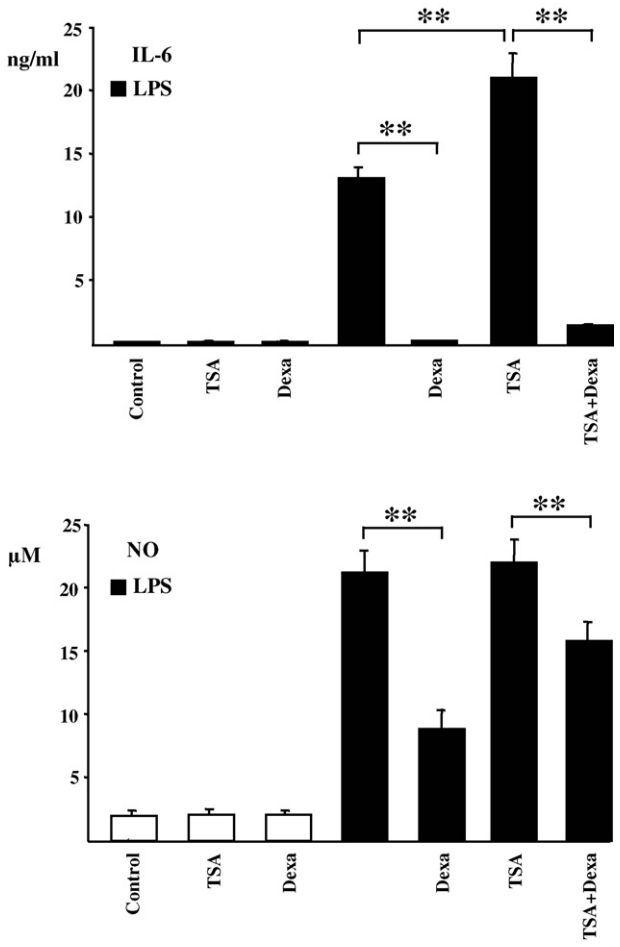

Fig. 4. Effects of dexamethasone on IL-6 and NO secretion induced by LPS itself or LPS and TSA together in rat hippocampal slices. Final concentrations were as in Fig. 2. Drug treatment was for 24 h. Values are means ± S.D. $^{**}p < 0.01$ (for the potentiation/inhibition).

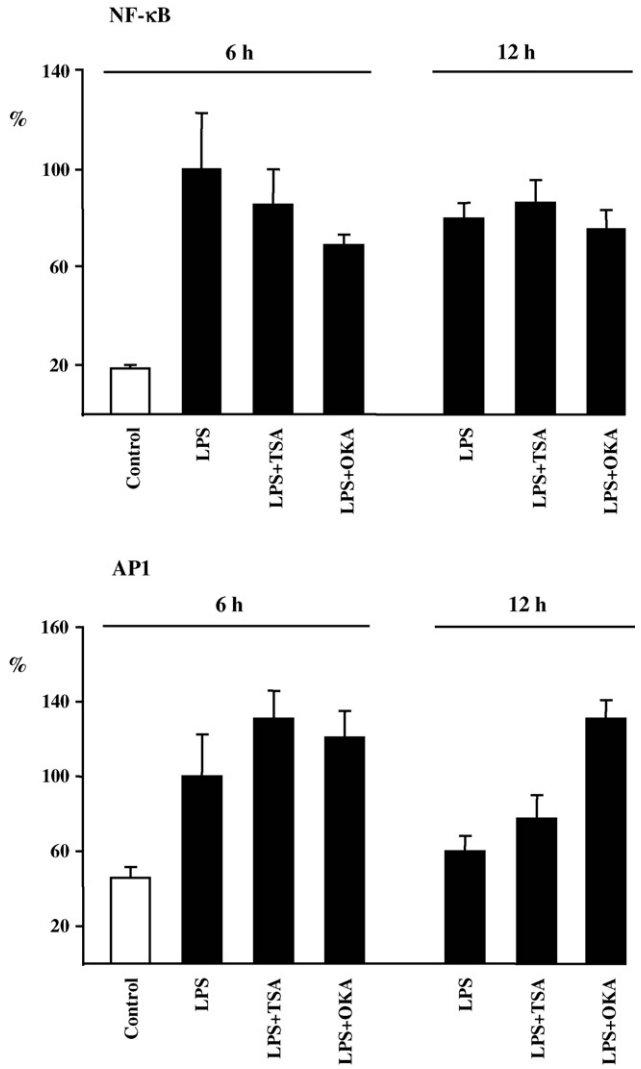

Fig. 5. Effects of LPS stimulation and TSA and okadaic acid (OKA) induced potentiation on the DNA-binding activities in EMSA of NF-κB and AP1 complexes in N9 microglia. Samples after 6 and 12 h exposures have been shown. DNA-binding values are calculated as pixel values of Storm PhosphorImager (see Section 2) and compared to the LPS response after 6 h (100% response). Values are means ± S.D. ($n = 3$). Experiment was repeated twice and this observation also verified earlier observations (Suuronen et al., 2003).

DNA-binding activity of NF-κB complex in N9 nuclear extract up to 6 h and this remained at the same level after 12 h. TSA or okadaic acid treatments did not enhance the nuclear DNA-binding activity although they both induced prominent potentiation of IL-6 secretion (Fig. 2). We have observed earlier that TSA exposure does not enhance DNA-binding activity of NF-κB even after a 24 h treatment period (Suuronen et al., 2003). LPS treatment also induced AP-1 binding activity but not to the same extent as NF-κB binding (Fig. 5). TSA exposure did not affect AP-1 binding activities but okadaic acid clearly enhanced AP1 binding activity after 12 h (Fig. 5). Our observations here and earlier (Suuronen et al., 2003) indicate that the DNA-binding activity of NF-κB and AP-1 factors does not regulate the enhancement of the inflammatory response during hyperacetylation in microglia.

3.5. Transactivation via NF-κB-Luc promoter is highly activated by TSA-induced pro-inflammatory potentiation

Next we studied whether the potentiation of the inflammatory response by protein hyperacetylation is attributable to the enhancement of transactivation of the NF-κB driven Luc reporter construct. We transiently transfected N9 microglia with the pNF-κB-Luc vector which contains multiple NF-κB sites at the enhancer region and with the negative control pTal-Luc plasmid (see Section 2). Fig. 6 shows that the control pTal-Luc vector showed only a minimal Luc reaction with all treatments but the NF-κB sites at the enhancer of Luc vector potentiated the responses. Interestingly, TSA treatment enhanced the expression of vector not only in the control samples but especially in the LPS treated N9 microglia (Fig. 6). Okadaic acid treatment did not evoke as extensive a response as seen after TSA exposure. If we compare these transactivation responses to the DNA-binding activities of NF-κB (Fig. 5), it seems that the increase in DNA-binding is necessary for the inflammatory response but the transactivation regulates the extent of the inflammatory response. Our studies also imply that

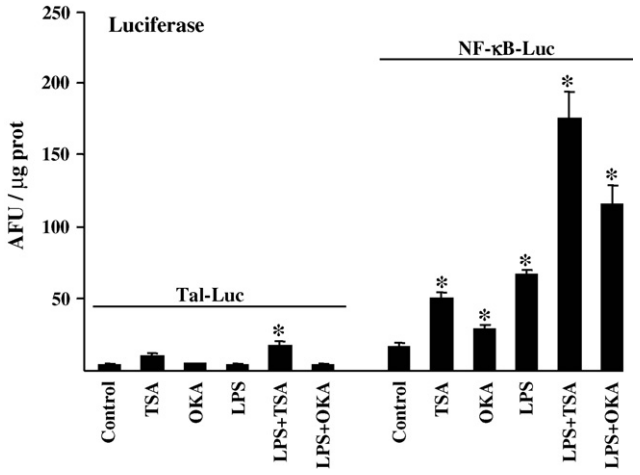

Fig. 6. Effects of LPS itself and LPS together with TSA or okadaic acid (OKA) on the transactivation of pTal-Luc (negative control) and pNF-κB driven Luc reporter gene in N9 microglia. Drug treatment was for 24 h. Final concentrations were as in Fig. 2. Values are means ± S.D. $^{*}p < 0.05$ (treated vs. control).

the potentiation of the inflammatory response by hyperacetylation stress is due to the enhancement of transactivation of NF-κB driven inflammatory genes.

3.6. PI3K inhibitor LY294002 blocks the TSA-induced pro-inflammatory response but does not affect the okadaic acid induced response

Several signaling pathways are able to regulate the LPS-induced inflammatory response (Palsson-McDermott and O'Neill, 2004). Next we focused on the signals which could either inhibit or even enhance the potentiated pro-inflammatory response mediated by protein hyperacetylation in N9 microglia. It is known that PI 3-kinase has an important role in the regulation of inflammation, probably it is a ''gatekeeper of inflammation'' upstream of NF-κB (Weaver and Ward, 2001; Ward et al., 2003). PI 3-kinase is a well-known activator of NF-κB signaling (Beraud et al., 1999) and the anti-inflammatory response induced by glucocorticoids is believed to be caused by the direct interaction of PI 3-kinase and the glucocorticoid receptor (Smoak and Cidlowski, 2004). Fig. 7A shows that a well-known inhibitor of PI 3-kinase, LY294002, inhibited the LPS-induced IL-6 secretion. Furthermore, LY294002 blocked the TSA-induced potentiation of IL-6 secretion. Interestingly, LY294002 did not affect the okadaic acid induced potentiation of IL-6 secretion in N9 microglia (Fig. 7A). This experiment was repeated several times and invariably LY294002 inhibited the pro-inflammatory response attributable to hyperacetylation but did not affect the response evoked by hyperphosphorylation. We also verified by using the MTT assay that the concentration of LY294002 used was not toxic to N9 microglia. Fig. 7B shows that TSA slightly increased the growth of N9 microglia in LPS-treated cells and LY294002 was not toxic to any of the samples.

We also verified that the inhibitory effect of LY294002 was not restricted to N9 microglia but occurred also in rat primary

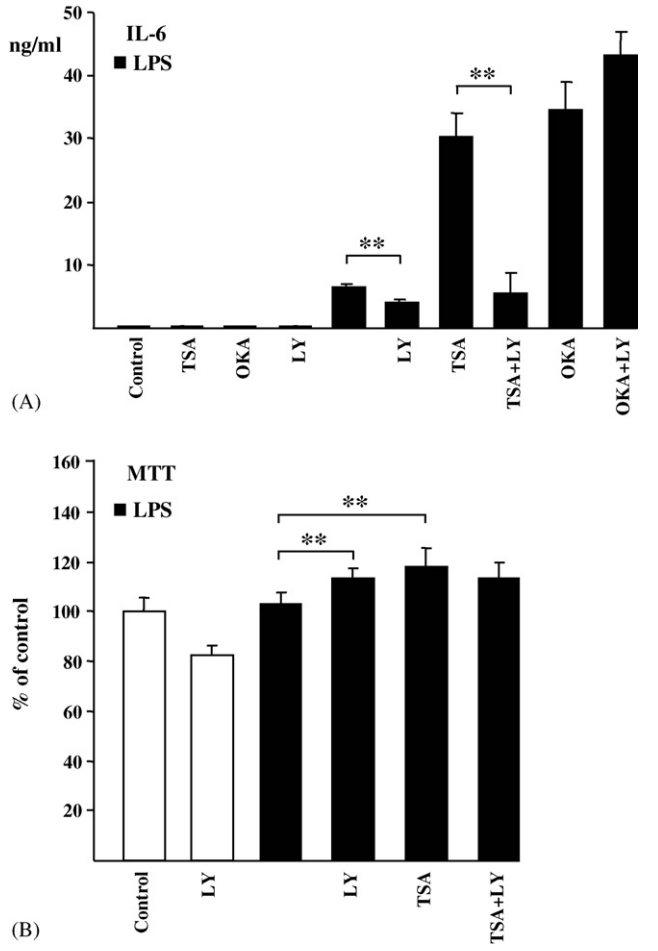

Fig. 7. Effects of LY294002 (LY) on IL-6 secretion induced by LPS itself and LPS together with TSA or okadaic acid (OKA) in N9 microglia (A). Final concentration of LY294002 was 10 μM and those of others as in Fig. 2. Drug treatment was for 24 h. 7B shows the viability values (MTT) of N9 microglia after different treatments for 24 h. Values are percent values from the control (100%). Values are means ± S.D. $^{**}p < 0.01$ (for the difference in IL-6 and MTT values).

hippocampal slice cultures. Fig. 8 shows that LY294002 strongly inhibited the LPS-induced increase in IL-6 and NO secretion but was even more effective at blocking the TSA-induced potentiation at IL-6 secretion.

We observed that wortmannin, another PI 3-kinase inhibitor, also inhibited the TSA-induced potentiation in the secretion of IL-6 and NO in N9 microglia at 500 nM concentration (data not shown). We focused on LY294002 since it caused a slightly more potent inhibition than wortmannin which also inhibits several other kinases.

To try to identify the regulatory pathways and inhibitors involved in the pro-inflammatory potentiation induced by hyperacetylation, we tested several well-known inhibitors of signal transduction. Inhibitors, such as H7 (10 μM, final conc.), H89 (1.0 μM), PMA (200 nM), JAK-3 inhibitor (1.0 μM), herbimycin (100 nM) and protein kinase Cζ pseudosubstrate inhibitor (1.0 μM) did not affect significantly ($p < 0.01$) the TSA-induced potentiation at non-toxic concentrations (data not shown).

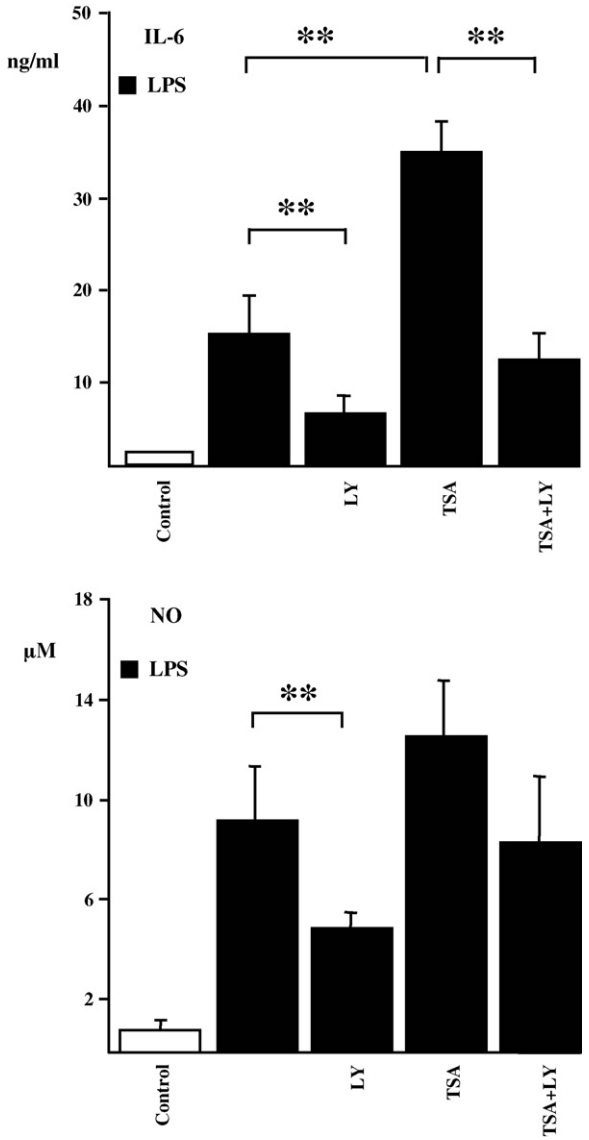

Fig. 8. Effect of LY294002 (LY) on IL-6 and nitric oxide (NO) secretion induced by LPS itself or LPS together with TSA in rat hippocampal slices. Final concentration of LY294002 was 10 μM and those of LPS and TSA as in Fig. 2. Drug treatment was for 24 h. Values are means ± S.D. $^{**}p < 0.01$ (for the potentiation/inhibition).

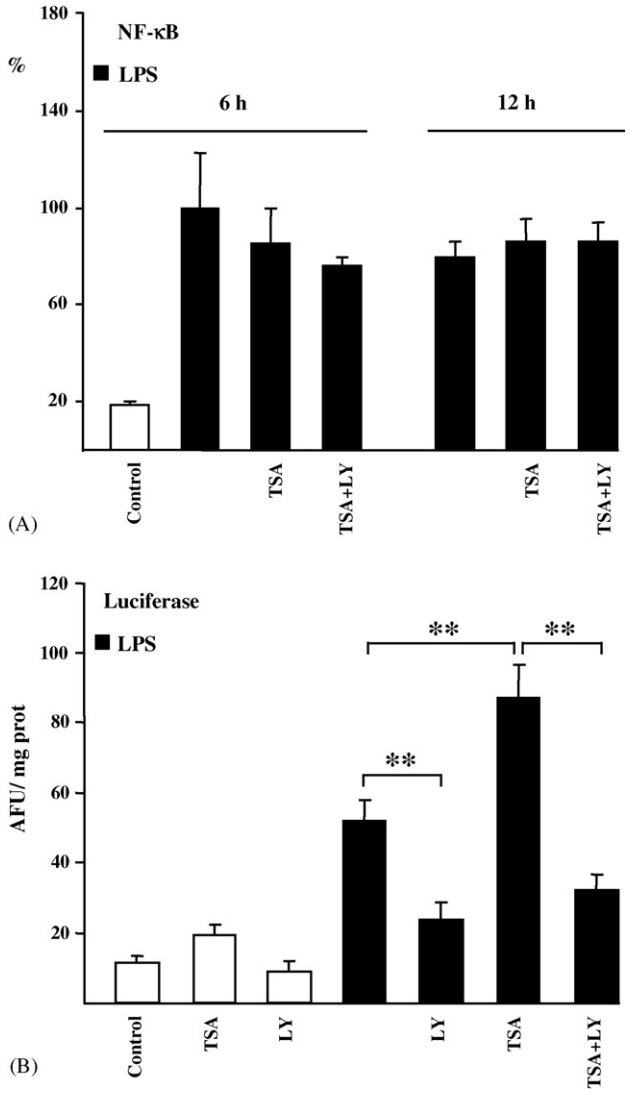

Fig. 9. Effect of LY294002 (LY) on the DNA-binding activity in EMSA of NF-κB complex (A) and transactivation of pNF-κB driven Luc reporter gene (B) in N9 microglia induced by LPS itself or LPS together with TSA. Drug concentrations were as in Figs. 2 and 7. Treatment time for transactivation was 24 h. Values are means ± S.D. $^{**}p < 0.01$ (for the potentiation/inhibition).

3.7. LY294002 does not inhibit DNA-binding activity of NF-κB but inhibits NF-κB-Luc reporter transactivation

Next we attempted to elucidate the mechanism to explain the ability of LY294002 to inhibit the pro-inflammatory response induced by hyperacetylation. We observed that LY294002 did not inhibit the DNA-binding activity of NF-κB in N9 microglia (Fig. 9A) which indicates that the translocation of NF-κB components is not affected. Subsequently we studied whether LY294002 could inhibit the transactivation of NF-κB-Luc reporter gene in N9 microglia. Fig. 9B shows that LY294002 clearly inhibited both the LPS-induced and the TSA-potentiated transactivation of the NF-κB-driven Luc reporter gene. These results suggest that PI 3-kinase regulates the transactivation efficiency of NF-κB-dependent transcription rather than transduction of NF-κB signaling.

4. Discussion

The significance of protein acetylation in signal processing has been compared to that of protein phosphorylation (Kouzarides, 2000) and both of these processes are also involved in the modification of inflammatory responses. Okadaic acid, an inhibitor of protein phosphatases PP2A and PP1, enhances protein phosphorylation in cells and simultaneously potentiates the inflammatory responses (Tebo and Hamilton, 1994; Shanley et al., 2001). The potentiation of inflammatory reactions is mediated via c-Jun N-terminal kinase (JNK) (Shanley et al., 2001; Avdi et al., 2002). Trichostatin A and several other histone deacetylase (HDAC) inhibitors, such as SAHA and M344, induce the acetylation of different cellular

proteins (Poledova and Sherman, 2002). Recent studies have shown that HDAC inhibitors clearly enhance inflammatory responses, for instance those occurring in microglial cells (Suuronen et al., 2003). Here we compared the potentiation of inflammatory responses induced by either TSA (acetylation response) or okadaic acid (phosphorylation response) in mouse N9 microglia stimulated by LPS. The time scale of the responses induced by these inhibitors revealed that the effects of okadaic acid are early-response effects which appear as soon as 6 h after exposure whereas at the same time TSA actually inhibited the LPS-induced response in IL-6 secretion. Up to the time point of 12 h, TSA caused a significant inhibition of IL-6 secretion but subsequently it induced an exponential increase in cytokine and nitric oxide production. It seems that okadaic acid induces early responses and TSA late but exponential events in microglial inflammatory responses. Furthermore, the effects are synergistic if the cells are exposed to both inducers at the same time, clear evidence that different signaling processes govern the acetylation and phosphorylation type of enhancers.

We have recently observed that TSA-induced potentiation can be blocked by the NF-κB inhibitors, helenalin and CAPE (Suuronen et al., 2003). Here we verified these effects using a more specific IKK-2 inhibitor, indicating that IKK-2 kinase is involved in both TSA and okadaic acid induced potentiation responses. Furthermore, we observed that dexamethasone, a steroidal anti-inflammatory drug, provides a profound inhibition against TSA and okadaic acid induced potentiation, as well as the LPS response itself. The anti-inflammatory response appeared both in N9 microglia and hippocampal slice cultures and was more prominent in terms of IL-6 secretion than in nitric oxide production. The molecular mechanisms of glucocorticoid receptor signaling during inflammation have been reviewed recently (Adcock et al., 2004; Smoak and Cidlowski, 2004). In addition to the repression of NF-κB transcription factors directly, glucocorticoid receptor signaling also interferes with the PI3K pathway, leading to the inhibition of NF-κB-mediated transcription (Beraud et al., 1999; Smoak and Cidlowski, 2004). Several studies show that PI 3-kinase possibly has a gatekeeper role in the regulation of inflammation upstream of NF-κB (Weaver and Ward, 2001; Ward et al., 2003). It is also known that PI3K mediates the activation signaling from TLR-4 to NF-κB (Guha and Mackman, 2001; Li et al., 2003).

Next we studied whether the TSA induced acetylation response and the okadaic acid induced phosphorylation response are both mediated via the PI3K pathway. Interestingly, we observed that LY294002, a specific inhibitor of PI3K, blocks only the TSA induced pro-inflammatory response but does not affect the okadaic acid induced response. We also verified that the inhibitory effect of LY294002 was not restricted to N9 microglial cells but also occurred in rat primary hippocampal slice cultures. Wortmannin, another inhibitor of PI3K, similarly inhibited the TSA-induced inflammatory potentiation. It seems that the TSA induced late inflammatory potentiation is mediated through the PI3K pathway via NF-κB activation whereas the okadaic acid induced early potentiation is mediated by the MAPK-JNK pathway (Guha and Mackman, 2001; Shanley et al., 2001; Avdi et al., 2002).

Subsequently, we examined whether TSA-induced potentiation is due to the activation of IKK in cytoplasm and the subsequent translocation of the NF-κB complex to nuclei to induce cytokine expression. We observed that LPS induced a prominent increase in the nuclear DNA-binding activity of the NF-κB complex already after 6 h of LPS exposure. Interestingly, TSA and okadaic acid treatments did not enhance the NF-κB binding capacity either after 6 or 12 h of exposure to LPS. This verifies our earlier observations (Suuronen et al., 2003). Furthermore, LY294002 did not affect the DNA-binding activity of the NF-κB complex during TSA-induced potentiation of IL-6 secretion. It seems that both TSA and okadaic acid do not regulate the inflammatory response at the cytoplasmic activation level but act at the transcriptional level by enhancing the transactivation efficiency of NF-κB complex.

The transactivation efficiency of NF-κB complexes was investigated by transfecting N9 cells with a pNF-κB-Luc reporter vector designed to monitor the activation of the NF-κB signaling pathway. Interestingly, although TSA did not affect the DNA-binding activity of NF-κB after LPS exposure, it strongly enhanced the transactivation potential of the NF-κB-driven reporter gene. Furthermore, LY294002 effectively blocked both the LPS induced and TSA potentiated responses. This suggests that TSA potentiates the inflammatory response in N9 microglial cells by increasing the transactivation capacity of NF-κB complexes. It is known that the site-specific acetylation of p65 component increases the transactivation capacity of NF-κB complex (Chen and Greene, 2003; Quivy and Van Lint, 2004; Rahman et al., 2004). Natoli et al. (2005) have shown that even the recruitment of NF-κB complex to the promoters of selected inflammatory genes is highly regulated and dependent on MAP kinases and histone acetylation (enhanced by TSA exposure). Avdi et al. (2002) have proposed a model where PP2A has a central role in the regulation of the c-Jun NH2-terminal kinase pathway. Hence okadaic acid induced inflammatory potentiation might be linked to the priming of chromatin to the NF-κB complex binding, since this response was inhibited by NF-κB inhibitor and dexamethasone. Furthermore, we observed that okadaic acid and TSA induced an additive pro-inflammatory response in N9 microglia. These observations indicate that the early inflammatory potentiation is protein phosphorylation dependent whereas the later phases are linked to protein acetylation and increased efficiency in NF-κB transactivation.

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