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SUPPRESSION BY DELTA-9-TETRAHYDROCANNABINOL OF LIPOPOLYSACCHARIDE-INDUCED AND INTRINSIC TYROSINE PHOSPHORYLATION AND PROTEIN EXPRESSION IN MOUSE PERITONEAL MACROPHAGES

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Abstract—Lipopolysaccharide (LPS, 100 ng/mL)-induced tyrosine phosphorylation of four proteins (p41, p42, p77, and p82) in mouse resident peritoneal macrophages was observed using a monoclonal anti-phosphotyrosine antibody PY20 immunoblotting method. Macrophages pretreated for 3 hr with 1 μg delta-9-tetrahydrocannabinol (THC)/mL had decreased tyrosine phosphorylation of p77 and p82 after incubation with LPS for 30 min. Simultaneous treatment of macrophages with THC (10 μg/mL) plus LPS for 30 min had a similar effect on p77 and p82 tyrosine phosphorylation. When the THC pretreatment protocol was combined with the simultaneous treatment protocol, 0.5 and 5 μg THC/mL, respectively, completely blocked LPS-induced p77 and p82 tyrosine phosphorylation. However, neither simultaneous treatment with THC nor pre- and simultaneous treatment had any effect on LPS-induced tyrosine phosphorylation of p41 and p42 in macrophages. Pretreatment with 1 μg THC/mL followed by simultaneous treatment with 10 μg THC/mL induced a p43 protein that showed tyrosine phosphorylation in place of p41 and p42. Further analysis of THC effects on macrophages revealed an increase in tyrosine phosphorylation as an immediate early event after THC treatment. Prolonged treatment of macrophages with THC resulted in a broad suppression of tyrosine phosphorylation and some cellular protein expression. Three cellular proteins (p65, p70, and p72) seemed most susceptible to inhibition by THC. The data suggest that suppression of tyrosine phosphorylation by THC in macrophages may be one of the mechanisms associated with inhibition of cell function, including the suppression of tumor necrosis factor-α release from macrophages.

Key words: macrophage; lipopolysaccharide; marijuana; tetrahydrocannabinol; tyrosine phosphorylation; signal transduction

Marijuana is a psychoactive, illegal drug commonly used throughout the world. Individuals who smoke marijuana have been reported to have decreased cellular immunity, as measured by uptake of tritiated thymidine in mixed lymphocyte cultures and in phytohemagglutinin responsiveness [1]. Various *in vitro* studies have shown that THC†, the major psychoactive component of marijuana, has a variety of inhibitory effects on immune functions [2, 3], including effects on macrophages [4]. THC was found to suppress macrophage spreading on plastic surfaces [5, 6], phagocytosis [5, 7], IL-1α [8] and TNF-α [9] production, and antiviral [10] as well as antibacterial [11] activity. Despite the important observations above, the mechanism by which THC suppresses macrophage functions is poorly understood.

Macrophages play a central role in host defenses to microorganisms and are major cellular targets for activation by bacterial LPS. TNF-α, which is principally secreted by activated macrophages, is an important mediator of both nonspecific and specific defenses against tumors and microbial infections [12]. We have found that exposure of macrophages to THC *in vitro* inhibits TNF-α production following LPS activation [9]. The activity of the drug is concentration dependent and is related to the amount of serum protein in the medium used to induce this cytokine [13]. However, the mechanism by which THC affects TNF-α production by LPS-activated macrophages remains unclear. Recent studies have reported that TNF-α production by LPS-activated macrophages involves LPS binding to the cell's receptor CD14 [14], tyrosine phosphorylation of cellular proteins [15], and NFκB induction [16]. We believe that the blockade of LPS-induced signal-transduction by THC may be involved in the suppression of macrophage function. In this report, THC treatment has been shown to interfere specifically with an LPS-induced early signaling event, i.e. tyrosine phosphorylation in mouse peritoneal macrophages. Moreover, THC was also found to inhibit nonspecifically intrinsic cellular

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† Abbreviations: THC, delta-9-tetrahydrocannabinol; LPS, lipopolysaccharide; IL-1α, interleukin 1-alpha; TNF-α, tumor necrosis factor alpha; NFκB, nuclear factor kappa B; IFN-γ, interferon gamma; TBS, Tris-buffered saline; TTBS, TBS containing 0.1% Tween 20; DAB, 3,3′-diaminobenzidine; IOD, integrated optical density; and G protein, guanine nucleotide-binding regulatory protein.

tyrosine phosphorylation and protein expression, using well defined culture conditions.

MATERIALS AND METHODS

Reagent preparations. RPMI 1640 (GIBCO, Grand Island, NY) medium containing 2 mM glutamine, 20 mM HEPES (pH 7.3), and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively) is referred to as "standard" medium. This medium when supplemented with LPS (100 ng/mL), mouse IFN-γ (100 U/mL; Genzyme, Cambridge, MA), and 0.5% BSA (Sigma Chemical Co., St. Louis, MO) is referred to as "induction" medium.

Phenol-extracted LPS from *Salmonella enteritidis* (Sigma Chemical Co.) was diluted in sterile pyrogen-free PBS to yield a stock concentration of 1 mg/mL, which was aliquoted and stored at −20°. Herbimycin A (Calbiochem, La Jolla, CA) was dissolved at 1 mg/mL in DMSO as a stock solution. After dividing the stock solution into aliquots, it was stored at −20°. Mouse anti-phosphotyrosine PY20 monoclonal antibody was purchased from ICN Biochemicals, Inc. (Costa Mesa, CA).

THC was obtained as an ethanol solution from the National Institute on Drug Abuse (NIDA), Bethesda, MD. The stock THC was dried to remove ethanol and then dissolved in DMSO to yield a stock concentration of 20 mg/mL as previously described [9].

Mouse macrophage preparation and treatment. Macrophages were obtained by washing the peritoneal cavity of 8- to 10-week-old female BALB/c mice with 5 mL of cold Hanks' balanced salt solution (HBSS) containing 50 mM HEPES. The cells were washed twice by centrifugation at 300 *g* for 10 min at room temperature and resuspended in standard medium. The cells were counted, adjusted to 2×10^6 cells/mL, and then plated in 6-well plates (Costar, Cambridge, MA) at 6×10^6 cells/well. After incubation for 2 hr at 37° in 5% CO_2 and 95% air, nonadherent cells were removed by washing with standard medium. Adherent cells were maintained in 4 mL of fresh standard medium, and THC treatment was performed as indicated in individual experiments. Then THC-treated and nontreated macrophage cultures were used for LPS stimulation or for cellular protein profile assay.

Anti-phosphotyrosine immunoblotting assay. Control or THC-treated peritoneal macrophages in 6-well plates were stimulated by LPS, LPS plus THC, or LPS plus herbimycin A in induction medium for the times indicated in each experiment. The cells were washed with ice-cold PBS containing 1 mM Na_3VO_4 and lysed in 0.25 mL of lysis buffer [20 mM Tris–HCl (pH 8.0), 137 mM NaCl, 10% glycerol (w/v), 1% Triton X-100, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin containing aprotinin at 0.15 U/mL] for 20 min at 4° [15]. This solution was added to block *in vitro* phosphorylation and dephosphorylation following cell lysis. The lysates were spun at 12,000 rpm in an Eppendorf centrifuge for 15 min at 4°. The supernatant fluids were collected and electrophoresed on a 12% SDS–PAGE gel until the bromophenol blue dye front ran off the gel (to improve protein separation). After electrophoresis, the gel was equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 30 min and then electrotransferred to pre-wet BIOTRANS PVDF membrane (ICN Biomedicals, Inc., Costa Mesa, CA) overnight at 20 V. After briefly washing in water, the blot membrane was blocked in TBS (10 mM Tris–HCl, 154 mM NaCl, pH 7.4) containing 3% BSA (Promega) and 1% ovalbumin (ICN) for 2 hr at room temperature. The blot membrane was washed three times with TTBS and then incubated with horseradish peroxidase-labeled monoclonal anti-phosphotyrosine antibody PY20 (1 μg/mL) [17], for 2 hr at room temperature. The blot membrane was washed again with TTBS three times with gentle agitation over 30 min and incubated with fresh DAB solution [50 mM Tris–HCl (pH 7.3), 0.03% nickel chloride, 0.06% DAB, 0.03% hydrogen peroxide] at room temperature until colored bands developed and a background began to be detectable. After removing the membrane from the container and washing it in another reservoir containing deionized water to halt color development, the blot membrane was air-dried and stored in a plastic bag in the dark for further analysis.

Cellular protein profile assay. Macrophage lysate preparation, SDS–PAGE, and electroblotting procedures were the same as above. The blot membrane was washed three times with TTBS and then stained using an India ink solution (ICN Biomedicals, Inc., 1:1000 in TTBS) for 30 min or longer until clear bands were visible with no dark background [18]. After washing away unbound ink in multiple changes of TBS for 30 min, the membrane was rinsed with water and air-dried.

Image analysis. Blots were analyzed further in a computerized Bio-image Scanner (Millipore, Bedford, MA). The scanner was standardized by a filter for reflected light provided by the company. The entire blot was photographed by a camera connected to the computer and then converted into a computer file. A whole band analysis program was used for each blot. The IOD of each band in each lane on each blot was chosen for final analysis.

RESULTS

LPS-induced tyrosine phosphorylation in mouse peritoneal macrophages. S. enteritidis LPS at 100 ng/mL was found to induce tyrosine phosphorylation of four Triton X-100-soluble proteins with apparent molecular masses of 41, 42, 77, and 82 kDa (p41, p42, p77, and p82) in BALB/c mouse peritoneal macrophages, in addition to numerous constitutively expressed proteins that were tyrosine phosphorylated (time 0) (Fig. 1). p42 and p82 were most prominent; thus, in some experiments these were the only newly phosphated bands observed. LPS-induced tyrosine phosphorylation of p77 and p82 was detectable within 5 min of LPS stimulation, reached a maximum by 30 min, and started to decline after 60 min. However, the LPS-induced tyrosine phosphorylation of p41 and p42 did not appear until later than that of p77 and p82. They were detectable at 30 min and appeared clearly by 60 min after LPS stimulation.

Effect of THC on LPS-induced tyrosine phos-

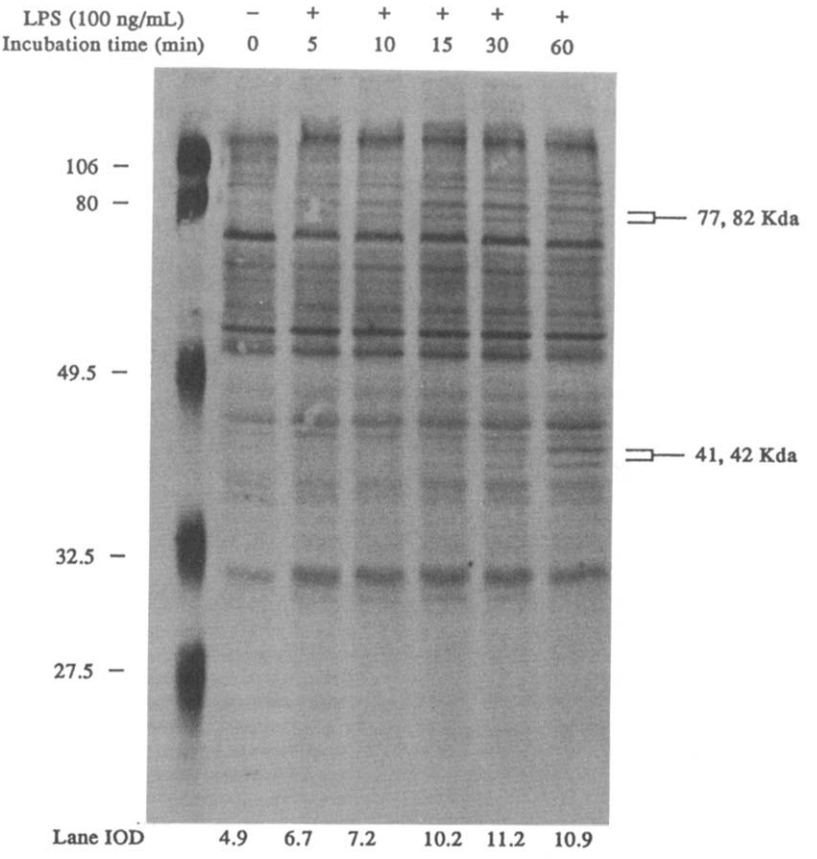

Fig. 1. Kinetics of tyrosine phosphorylation induced by LPS. LPS-induced protein tyrosine phosphorylation in resident peritoneal macrophages was shown by anti-phosphotyrosine immunoblots of Triton X-100-soluble proteins. Macrophages at 6×10^6 cells/well in a 6-well plate were cultured in standard RPMI 1640 medium for 3 days with daily medium changes and then subjected to stimulation by induction medium [RPMI 1640 with LPS (100 ng/mL), IFN-γ (100 U/mL), and 0.5% BSA] for the indicated time. At different time points, the stimulated cells were washed *in situ* with ice-cold PBS containing 1 mM Na_3VO_4 and then were lysed in lysis buffer. An equal amount (37.5 μL) of cell lysates from different samples was separated by 12% SDS–PAGE, transferred to BIOTRANS PVDF membrane, and probed with anti-phosphotyrosine monoclonal antibody PY20 (1 μg/mL)-horseradish peroxidase conjugate. Diaminobenzidine was used to develop the immunoreactive bands. Then the entire blot was scanned and analyzed using a Bio-image system. The blot represents results from one of four experiments.

phorylation in murine macrophages. Macrophages pretreated for 3 hr with THC at 0.1, 0.5 or 1 μg (3.2×10^{-6} M)/mL showed decreased tyrosine phosphorylation of p82 after incubation with LPS for 30 min (Fig. 2). Inhibition by THC at 0.1 μg/mL was 8.5%, at 0.5 μg/mL, 18%, and at 1 μg/mL, 70% compared with LPS-induced p82 tyrosine phosphorylation in the absence of THC, when measured by image analysis. When macrophages were pretreated with 0.5 μg THC/mL and treated with 5 μg THC/mL simultaneously with LPS stimulation, tyrosine phosphorylation of p77 and p82 was inhibited markedly whereas the phosphorylation of other cellular proteins was not affected (Fig. 3). However, the macrophages that received a combination treatment of THC (1 μg/mL for pretreatment and 10 μg/mL for simultaneous treatment) had a general suppression of cellular protein tyrosine phosphorylation. In this case, the tyrosine phosphorylation of p77 and p82 was abrogated completely. Similarly, simultaneous treatment of macrophages with 10 μg THC/mL and LPS (100 ng/mL) for 30 min strongly blocked the tyrosine phosphorylation of p77 and p82, but did not interfere with other cellular protein tyrosine phosphorylation (Fig. 3). Under both of these experimental conditions, herbimycin A, a tyrosine kinase inhibitor, was included as a positive control for inhibition of tyrosine phosphorylation. This inhibitor showed specific inhibition of LPS-induced tyrosine phosphorylation (Figs 2 and 3), confirming that the THC effect was on tyrosine phosphorylation. It was clear that inhibition of tyrosine phosphorylation of p77 and p82 was THC concentration dependent. However, inhibition appeared to be selective (Figs. 2 and 3) since THC treatment of macrophages did not show an obvious decrease of LPS-induced tyrosine phosphorylation of p42 until a general

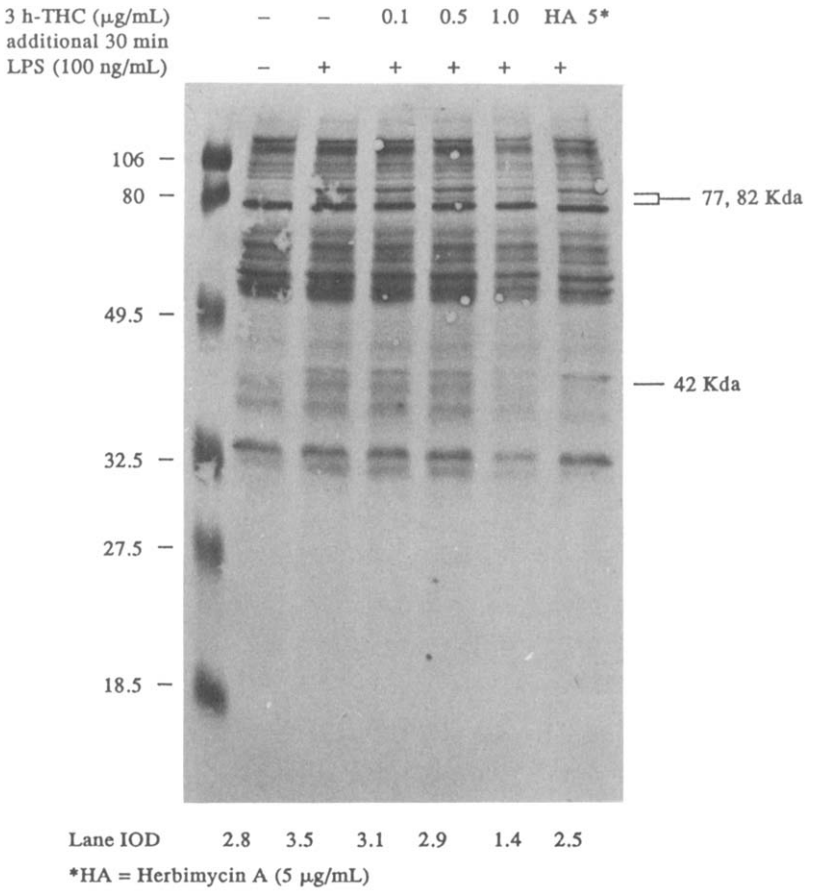

Fig. 2. Effect of THC pretreatment on LPS-induced tyrosine phosphorylation in macrophages. Macrophages at 6×10^6 cells/well in a 6-well plate were cultured for 2 days in standard RPMI 1640 medium and then incubated with or without THC in standard RPMI 1640 medium for 3 hr prior to a 30-min stimulation in induction medium. The stimulated macrophages were then washed, lysed and stained as indicated in Fig. 1. The blot represents results from one of three experiments.

suppression appeared (Figs 2 and 3). A combination of THC pretreatment and simultaneous (with LPS) treatment for 1 hr for each concentration again showed no significant decrease in the p42 tyrosine phosphorylation band. However, tyrosine phosphorylation of a 43 kDa protein was induced by combination treatment (1 μg THC/mL pretreatment and 10 μg THC/mL simultaneous treatment), although the generalized tyrosine phosphorylation of protein in these cells was only 42% of the LPS control (Fig. 4).

Effect of THC on intrinsic tyrosine phosphorylation and protein expression. Macrophages were treated with THC alone (no LPS) in these experiments in order to demonstrate that THC both specifically inhibits LPS-induced tyrosine phosphorylation and nonspecifically affects intrinsic tyrosine phosphorylation, as seen in Figs. 2–4. Results indicated that THC treatment at 1 μg/mL increased tyrosine phosphorylation after 15, 30, and 60 min of incubation but decreased tyrosine phosphorylation when the treatment was prolonged to 2–3 hr at 37° (Fig. 5). However, THC *per se* was unable to induce a new tyrosine-phosphorylated protein in the cells.

Figure 6 shows that the effect of THC on the profile of proteins in macrophage lysates is similar to that on cellular protein tyrosine phosphorylation. THC slightly inhibited (24%) expression of three proteins (p65, p70, and p72) after 3 hr of incubation at 37°, whereas total cellular protein was about 88% of the control at this time point. Thus, p65, p70, and p72 were more susceptible to THC inhibition than were other cellular proteins (24 vs 12%). When increasing concentrations and combinations of THC treatment were used, inhibition became more evident. Using a combination of 3-hr THC treatment (1 μg/mL) with an additional 15-min THC treatment (10 μg/mL) in the presence of LPS (Fig. 7), cellular p65, p70, and p72 levels were only 60, 23, and 5% of the control, respectively. There was no difference in the protein profile before and after LPS stimulation for 15 min. However, some proteins were unaffected, and total cellular protein expression in macrophages was generally suppressed to a more limited extent (67% of control) by this THC treatment (Fig. 7).

DISCUSSION

The activation by LPS of macrophages has been

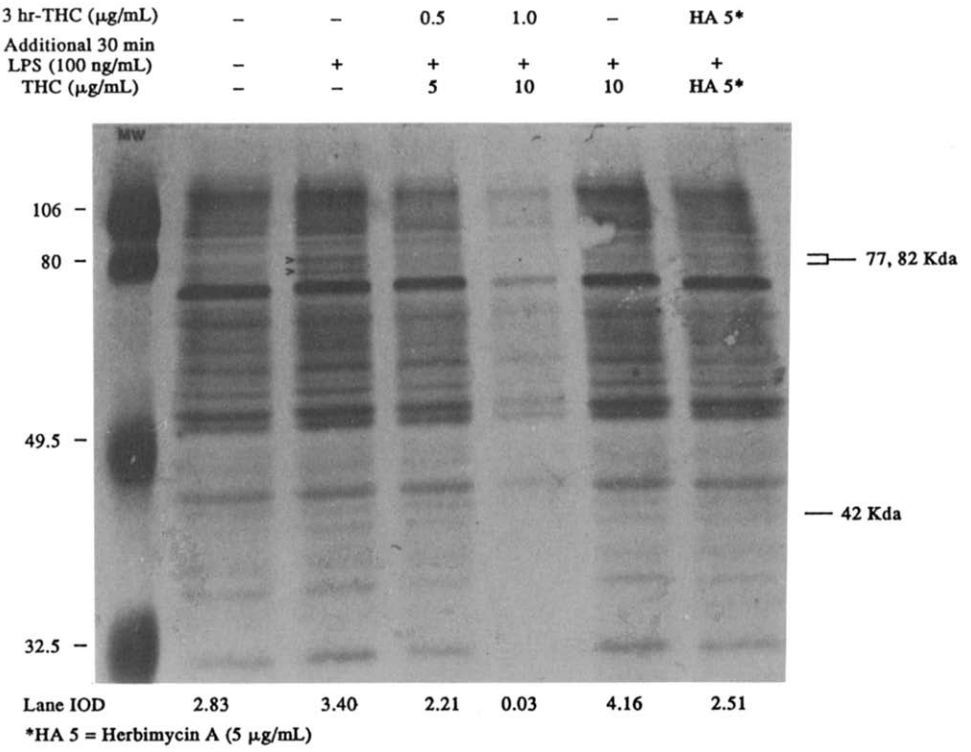

Fig. 3. Effect of THC pretreatment and/or simultaneous treatment on LPS-induced p77 and p82 tyrosine phosphorylation in macrophages. Macrophages in these experiments were cultured for 3 days in standard RPMI 1640 medium and then treated with or without THC or herbimycin A in standard RPMI 1640 medium for 3 hr. Thereafter, all macrophage cultures were stimulated for 30 min using induction medium with or without THC or herbimycin A, as indicated. The rest of the protocol was as described in the legend of Fig. 1. The blot represents results from one of three experiments.

associated with binding of LPS to a cell surface receptor, which initiates a signal transduction pathway involving a G protein [19], protein kinase C [20], and the phosphotyrosine kinase pathway [15, 21]. The present report provides evidence to show that tyrosine phosphorylation, an important signaling pathway during LPS-stimulated macrophage activation, is inhibited by THC. Four proteins, p41, p42, p77, and p82, were identified in our *in vitro* model as LPS-induced proteins that undergo tyrosine phosphorylation. Kinetic studies on LPS-induced tyrosine phosphorylation of the four proteins showed that phosphorylation is an early event and appears sequentially: p77 and p82 were detected by 5 min after LPS stimulation, whereas p41 and p42 did not appear until 30 min after LPS stimulation, at a time when p77 and p82 reach their peak levels. In addition, higher levels of tyrosine phosphorylation of p41 and p42 were found associated with declining tyrosine phosphorylation of p77 and p82. Thus, this cascade of tyrosine phosphorylation might indicate that these proteins are involved in intracellular signal transduction similar to the mitogen-activated protein kinase (MAPK) pathway [22]. Since LPS-induced tyrosine phosphorylation of p41 and p42, as well as p77 and p82, was sometime inseparable in SDS–PAGE, it seems that p41 and p77 may be somehow related to p42 and p82, respectively, during LPS-stimulated tyrosine phosphorylation. The significance of these relationships is unclear at present. Although our induction medium contained IFN-γ to be consistent with previously reported induction conditions [9, 13], IFN-γ, by itself, did not induce tyrosine phosphorylation of protein in these (data not shown) or in other [21] studies. Furthermore, IFN-γ did not stimulate macrophages to release TNF-α in these experiments (unpublished observations) or in other reports [23]. Thus, the observed results imply that LPS is responsible for induction of tyrosine phosphorylation, but LPS activity, in general, is boosted by IFN-γ.

It is well known that many receptors stimulate tyrosine phosphorylation of protein following ligand binding, and this event is thought to be part of the signal-transduction mechanism that mediates later cellular responses [24, 25]. It has been proposed that activation of macrophages by LPS is initiated by LPS binding to either CD14 [14] or an 80 kDa LPS receptor [26] on the macrophage surface. The present study shows that an LPS-induced tyrosine-phosphorylated p82 has a molecular weight close to that of the LPS p80 receptor [26]. The addition of THC to medium used to maintain macrophage cultures resulted in suppression of LPS-induced tyrosine phosphorylation of p77 and p82. The suppression was THC concentration dependent and

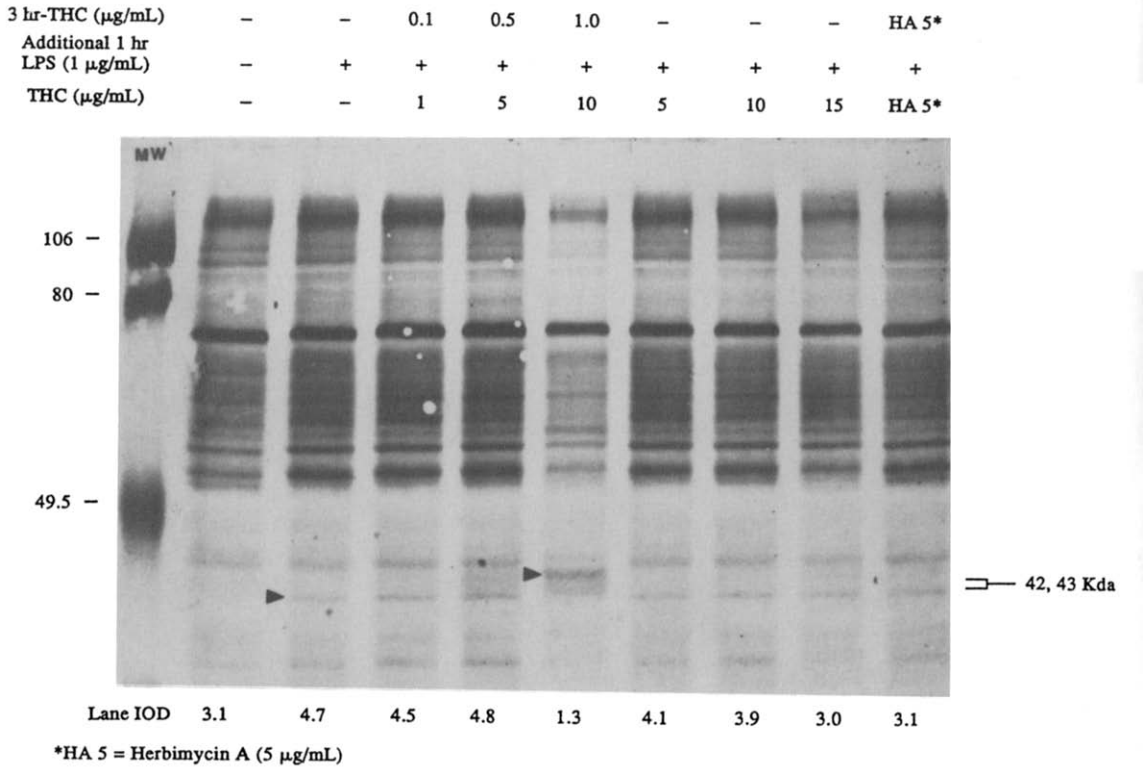

Fig. 4. Effect of THC pretreatment and/or simultaneous treatment on LPS-induced p42 and p43 tyrosine phosphorylation. The experimental protocol was exactly the same as described in the legend of Fig. 3 except that a higher dose of LPS (1 μg/mL) was used in the induction medium and a longer induction time (1 hr) was used. The blot represents results from one of two experiments.

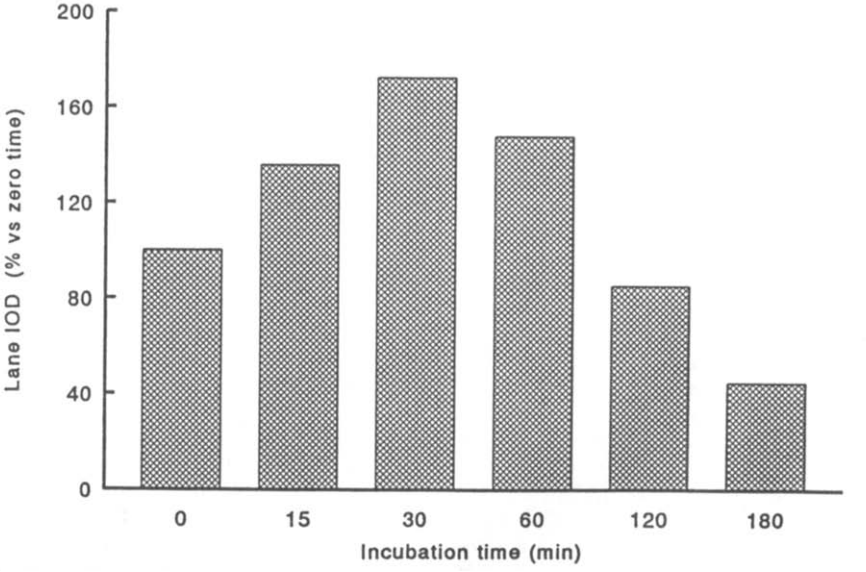

Fig. 5. Kinetic effect of THC on intrinsic tyrosine phosphorylation. Macrophages were cultured in standard RPMI 1640 medium for 3 days at 37° and then subjected to THC treatment (1 μg/mL) for the indicated time. The macrophages were then washed, lysed, and assayed as described in the legend of Fig. 1. The integrated optical density of each band in each lane was accumulated and compared with the sample at zero time. The blot represents results from one of three experiments.

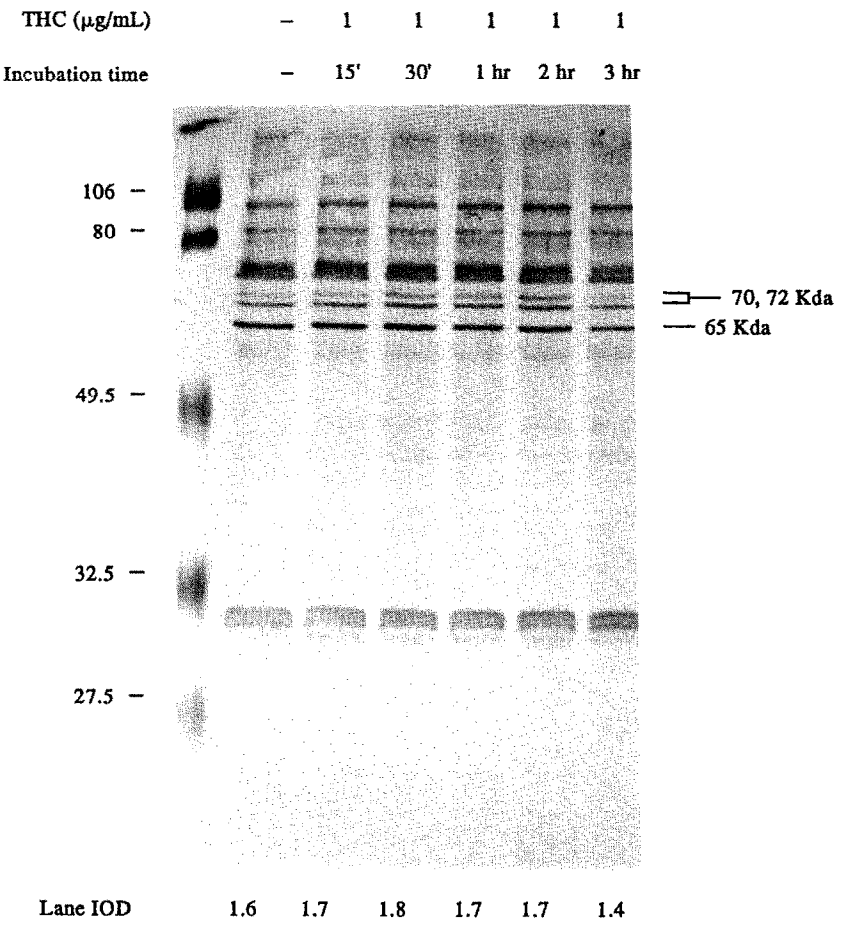

Fig. 6. Kinetic effect of THC on protein expression in macrophages. This experiment was very similar to that in Fig. 5 except that the protein bands on a BIOTRANS PVDF membrane were directly stained with India ink after electrotransferring overnight (see Materials and Methods). The lane IOD indicated at the bottom for each sample was shown for comparison of protein content. The blot represents results from one of three experiments.

was related to the kinetics of the treatment protocols. Treatment of macrophages with THC prior to LPS stimulation was important because LPS-induced tyrosine phosphorylation occurred within 5 min of LPS stimulation. Since THC is lipophilic and can be incorporated into cell membranes [27], it is possible that THC pretreatment of macrophages may affect cell membrane proteins, including LPS receptors, resulting in the decreased LPS-induced tyrosine phosphorylation. In addition, it is possible that the signal transduction cascade was inhibited at any stage from G protein binding to protein kinase C activation. Several reports have shown that THC can alter the expression of several products involved in immune cell activation [28, 29]. The 3-hr THC pretreatment protocol was chosen based upon our previous work in which this protocol significantly blocked LPS-induced TNF-α production by macrophages [9]. The greatest inhibition of tyrosine phosphorylation of p77 and p82 was the use of combination treatment (3-hr THC pretreatment plus simultaneous addition of THC and LPS). However, this protocol did not result in inhibition of LPS-induced tyrosine phosphorylation of p41 and p42 (Figs. 3 and 4) until a high THC concentration resulted in a general suppression. Interestingly, this combination THC treatment for 1 hr induced tyrosine phosphorylation of a 43 kDa protein. This could be explained as an isoform of p42 or as a result of degradation of the larger tyrosine phosphorylated proteins by THC or an induced product. The higher dose (1 μg/mL) (Fig. 4) and 1-hr stimulation of LPS was chosen in order to have more tyrosine phosphorylation of p41 and p42. Okuda *et al.* [30] indicate that insulin can induce a 43 kDa isoform of $p42^{mapk}$ that is a protein kinase. Whether this is similar to the finding reported here remains to be determined. Nevertheless, the mechanism by which THC alters macrophage tyrosine kinase activity requires further investigation.

It has been reported that THC inhibits macromolecular synthesis in a variety of cell cultures [31–34] including lymphocytes [35] and macrophages [36]. The present study also showed that incubation

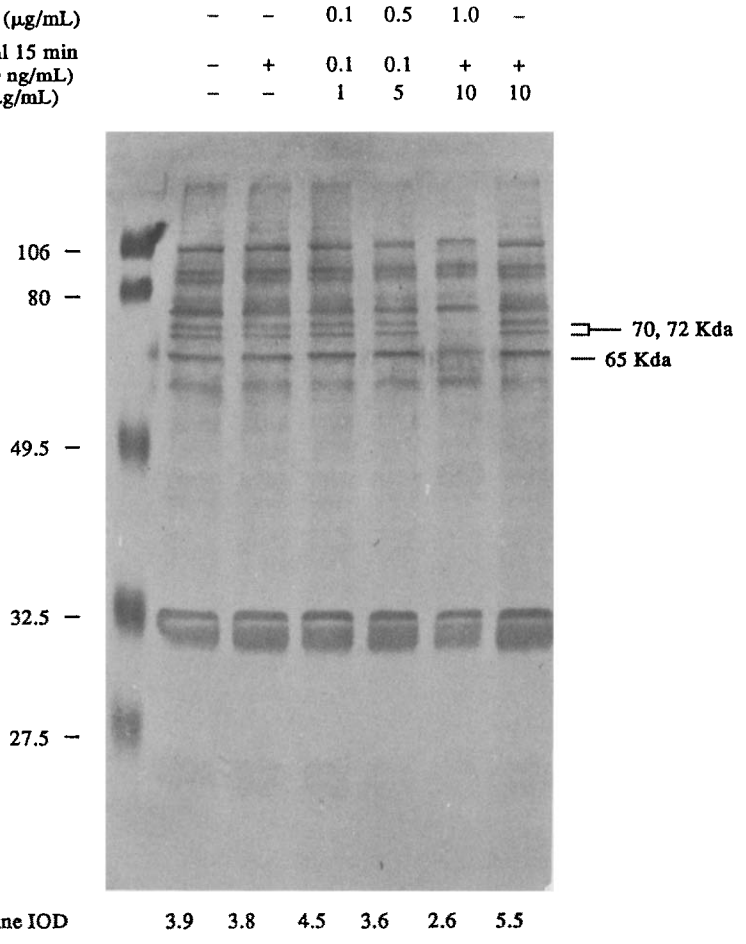

Fig. 7. Effect of combined THC treatment on protein expression in macrophages. Macrophages at 6×10^6 cells/well in 6-well plates were cultured for 3 days in standard RPMI 1640 medium. Then the cultured macrophages were incubated with or without THC in RPMI 1640 for 3 hr at the indicated concentrations similar to the THC pretreatment protocol for the tyrosine phosphorylation studies. Thereafter, all macrophage cultures were treated again with LPS (100 ng/mL) in standard RPMI 1640 medium supplemented with THC, as indicated, for another 15 min. The macrophage cultures were washed and lysed as described in the legend of Fig. 1. The cell lysates were separated by 12% SDS–PAGE, transferred to a BIOTRANS PVDF membrane, stained with India ink, and scanned using a Bio-image system. The blot represents results from one of two experiments.

with 1 μg THC/mL for 3 hr suppressed cellular protein expression as well as tyrosine phosphorylation of protein in murine resident macrophages. However, short-term incubation (30–60 min) of macrophages in medium containing 1 μg THC/mL can stimulate tyrosine phosphorylation of cellular proteins. This enhanced macrophage reaction to THC seems to be an immediate early event and remains to be elucidated. The inhibition by THC of intrinsic tyrosine phosphorylation of cellular proteins occurred following a 2- hr THC pretreatment (Fig. 5), whereas the suppression of cellular protein expression required a 3- hr THC treatment at a concentration of 1 μg/mL (Fig. 6). The results suggest that general blockade by THC of cellular protein expression could be due to the inhibition of cellular signal transduction, as suggested by Schatz *et al.* [37]. In addition to the general suppression of cellular protein expression, THC has been shown to more specifically inhibit p65, p70, and p72. The reason that these three proteins appeared more susceptible to THC inhibition and how this is related to the inhibition by THC of tyrosine phosphorylation remain to be determined.

In summary, LPS can stimulate tyrosine phosphorylation in mouse resident peritoneal macrophages. This reaction of macrophages to LPS stimulation could be blocked by pretreatment with low concentrations of THC and/or simultaneous treatment with high concentrations of THC. By contrast, the immediate early response of macrophages to THC is an increase in tyrosine phosphorylation. High THC concentrations and/or prolonged treatment of macrophages with THC result in moving from increased to suppressed tyrosine phosphorylation in macrophages. This pro-

gression from hyperactivity to hyporesponsiveness is reminiscent of T cell responsiveness to superantigens [38, 39] and may be a common cellular response to external stimuli. Thus, THC suppression of tyrosine phosphorylation in macrophages may be one of the mechanisms that is responsible for inhibition of macrophage functions, including the suppression of TNF-α release from macrophages [9].

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