Cellular/Molecular

Production and Release of Neuroprotective Tumor Necrosis Factor by $P2X_7$ Receptor-Activated Microglia

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After a brain insult, ATP is released from injured cells and activates microglia. The microglia that are activated in this way then release a range of bioactive substances, one of which is tumor necrosis factor (TNF). The release of TNF appears to be dependent on the $P2X_7$ receptor. The inhibitors 1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]butadiene (U0126), anthra[1,9-cd]pyrazol-6(2H)-one (SP600125), and 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)IH-imidazole (SB203580), which target MEK (mitogen-activated protein kinase kinase), JNK (c-Jun N-terminal kinase), and p38, respectively, all potently suppress the production of TNF in ATP-stimulated microglia, whereas the production of TNF mRNA is strongly inhibited by U0126 and SP600125. SB203580 did not affect the increased levels of TNF mRNA but did prevent TNF mRNA from accumulating in the cytoplasm. The ATP-provoked activation of JNK and p38 [but not extracellular signal-regulated kinase (ERK)] could be inhibited by brilliant blue G, a $P2X_7$ receptor blocker, and by genistein and 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-D]pyrimidine, which are general and *src*-family-specific tyrosine kinase inhibitors, respectively. Most important, we found that treatment of the microglia in neuron–microglia cocultures with the $P2X_7$ agonist 2′–3′-*O*-(benzoyl-benzoyl) ATP led to significant reductions in glutamate-induced neuronal cell death, and that either TNF-α converting enzyme inhibitor or anti-TNF readily suppressed the protective effect implied by this result. Together, these findings indicate that both ERK and JNK are involved in the regulation of TNF mRNA expression, that p38 is involved in the nucleocytoplasmic transport of TNF mRNA, and that a PTK (protein tyrosine kinase), possibly a member of the *src* family, acts downstream of the $P2X_7$ receptor to activate JNK and p38. Finally, our data suggest that $P2X_7$ receptor-activated microglia protect neurons against glutamate toxicity primarily because they are able to release TNF.

Key words: **$P2X_7$ receptor; TNF; MAP kinase; ATP; microglia; neuroprotection**

Introduction

ATP is released from damaged cells as a result of ischemia or inflammation and serves as a cell-to-cell mediator through cell surface P2 receptors, which are widely distributed throughout the nervous system (Inoue, 2002). P2 receptor subfamilies have been divided into two subtypes: P2X and P2Y. P2X receptors ($P2X_1$–$P2X_7$) are coupled to nonselective cation channels, allowing influx of Na^+ and Ca^{2+}, leading to transient cell depolarization, whereas P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$) are G-protein coupled, and their activation leads to inositol lipid hydrolysis, intracellular Ca^{2+} mobilization, or modulation of adenylate cyclase activation (North and Surprenant, 2000; Sak and Webb, 2002). Microglia have been shown to express multiple P2 receptor subtypes, including $P2X_7$, $P2Y_2$, and $P2Y_{12}$ (Norenberg et al., 1994; James and Butt, 2002), indicating that ATP may be a critical regulator of microglial cell function. In fact, ATP strongly induces microglial chemotaxis via the G_i- and G_o-coupled $P2Y_{12}$ receptor (Honda et al., 2001) and stimulates the release of plasminogen, interleukin-6 (IL-6), and IL-1β (Ferrari et al., 1997; Inoue et al., 1998; Shigemoto-Mogami et al., 2001) by means of different types of P2 receptor and intracellular signals. ATP also stimulates the *de novo* synthesis and release of tumor necrosis factor (TNF) from rat microglia that flows from the activation of $P2X_7$ receptors, extracellular signal-regulated kinase (ERK), and p38 mitogen-activated protein (MAP) kinase (Hide et al., 2000). The precise roles of each of these MAP kinases in $P2X_7$ receptor-mediated TNF release have not yet been determined. Moreover, although the ATP-mediated activation of ERK and p38 by ATP occurs in the absence of extracellular Ca^{2+}, it is still not known precisely how MAP kinases are activated via $P2X_7$ receptors.

TNF is a proinflammatory cytokine that is upregulated in the brain in response to various insults or injury. This cytokine is mainly expressed by microglia and astrocytes around the injured area. The function of TNF, however, remains controversial.

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Although TNF has been implicated in the acceleration of injury and the pathology of neurodegenerative diseases, recently emerging evidence suggests that TNF may also serve a protective role. The precise mechanisms involved in these two seemingly conflicting activities are still undetermined, as is the role of the TNF that is released by $P2X_7$ receptor-activated microglia.

In the present study, we investigated the signaling mechanism by which the activation of microglial $P2X_7$ receptors regulates TNF release via ERK, JNK (c-Jun N-terminal kinase), and p38 MAP kinase. We also examined whether the TNF released by microglia is toxic or protective for neurons. Our data demonstrate that ERK, JNK, and p38 contribute in different ways to the ATP-provoked production of TNF. In addition to outlining a new mechanism for the activation of JNK and p38 via the $P2X_7$–src-family protein tyrosine kinase pathway, we provide the first evidence that $P2X_7$ receptor-activated microglia protect neurons against glutamate neurotoxicity because they release TNF.

Materials and Methods

Reagents. All reagents for cell culture, ATP, and 2′–3′-*O*-(benzoylbenzoyl) ATP (BzATP) were obtained from Sigma (St. Louis, MO), and a rat TNF ELISA kit was obtained from Biosource International (Camarillo, CA). Antibody kits for p42–p44 (ERK), JNK, and p38 MAP kinase were obtained from Cell Signaling Technology (Beverly, MA). All other reagents were purchased from commercial sources and were of the highest available purity. We checked all reagents used for endotoxin contamination.

Cell culture. Microglia were obtained from primary cell culture of neonatal rat brains as described previously (Nakajima et al., 1989, 1992). After 7–16 d in culture, microglia were prepared as a floating cell suspension. Aliquots ($1.5–2.0 \times 10^5$ cells) were transferred to the wells of a 24 well plate and allowed to adhere at 37°C for 45 min. Unattached cells were removed by rinsing with serum-free DMEM. Neuron-enriched cultures were prepared from primary cell culture of neonatal rat brain cortex. For neuron–microglia cocultures, cortical neurons were seeded into 24 well plates, and microglia were transferred in Transwell coculture inserts (Costar, Cambridge, MA).

TNF assay. For the assay of TNF release, microglia were incubated with 0.4 ml of serum-free DMEM with or without drugs for 3 hr. For the assay of intracellular TNF, microglia were incubated for 2 hr and then solubilized with 0.1% Triton X-100. TNF was assayed in 50 μl samples using a rat TNF ELISA kit according to the instructions of the manufacturer.

RNA extraction. Microglia were plated in 60 mm dishes (7.5×10^5 cells per 3 ml per dish) and stimulated with ATP or the $P2X_7$ agonist BzATP for 1 hr. The cells were dissolved in Trizol LS reagents (Invitrogen, Gaithersburg, MD) before total RNA extraction. Briefly, samples were ethanol precipitated, the pellets were dissolved in RNase-free water, and RNA concentrations were determined spectrophotometrically.

Real-time reverse transcription-PCR assay. Total TNF mRNA was measured by real-time quantitative reverse transcription (RT)-PCR (ABI Prism model 7700 sequence detection system; PerkinElmer Applied Biosystems, Foster City, CA). RT-PCR was performed using the TaqMan one-step RT-PCR master mix reagents kit according to the protocol of the manufacturer (PerkinElmer Applied Biosystems). The sequences of the forward and reverse primers were 5′-ACAAGGCTGCCCCGACTAC-3′ and 5′-TCCTGGTATGAAATGGCAAACC-3′, respectively. The TaqMan fluorogenic probe was 5′-6FAM-TGCTCCTCACCCACACCGTCAGC-TAMRA-3′. During PCR amplification, 5′ nuclease activity of AmpliTaq Gold DNA polymerase cleaves the TaqMan probe, separating the 5′ reporter dye from 3′ quencher dye, resulting in increased fluorescence of the reporter. Reaction conditions for PCR were as follows: 40 cycles of amplification by denaturing to 95°C for 15 sec and extending at 60°C for 1 min. The threshold cycle, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level. The TNF mRNA levels were corrected for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA using a VIC probe according to the protocol of the manufacturer.

Western blot analysis. Western blots were performed for the analysis of ERK (p44 and p42), JNK, and p38 activation using a MAP kinase antibody kit according to the protocol of the manufacturer. In brief, the cells were washed with PBS, lysed by adding SDS sample buffer, and sonicated. After heating to 95°C for 5 min, the protein samples were separated by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer containing 3% skim milk for 3 hr at room temperature and incubated with primary antibody with gentle agitation overnight at 4°C. After washing, the membranes were incubated for 1 hr at room temperature with horseradish peroxidase-conjugated secondary antibody and horseradish peroxidase-conjugated anti-biotin antibody to detect biotinylated protein markers. The membranes were then washed and incubated with Lumi GLO, and the proteins were detected by exposure to x-ray film.

Viability assay. The viability of neurons was assessed by a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay on the basis of the reduction of the tetrazolium salt MTT by the mitochondrial dehydrogenase in living cells.

Statistical analysis. Student's *t* test was used in all determinations of statistical significance.

Results

The roles of ERK, JNK, and p38 MAP kinase in TNF mRNA expression and the production and release of TNF in ATP-stimulated microglia

ATP rapidly activates ERK, JNK, and p38 MAP kinase in microglia. To investigate possible roles of ERK, JNK, and p38 in ATP-induced TNF release, we examined the inhibitory effects of 10 μM 1,4-diamino-2,3-dicyano-1,4-bis[2-amino-phenylthio]-butadiene (U0126) [which inhibits MEK (mitogen-activated protein kinase kinase)], 30 μM anthra[1,9-cd]pyrazol-6(2H)-one (SP600125) (which inhibits JNK), and 15 μM 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)IH-imidazole (SB203580) (which inhibits p38) on the release of TNF from ATP-stimulated microglia. All three inhibitors proved to be capable of reducing the amounts of TNF released by ATP-treated microglia very significantly, suggesting that TNF release is dependent on the activities of all three MAP kinases (Fig. 1*A*). To confirm the supposed selectivity of these particular inhibitors, we conducted experiments with the two additional inhibitory compounds 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) (which inhibits MEK) and 4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)IH-imidazole (SB202190) (which inhibits p38) and found that 25 μM PD98059 and 10 μM SB202190 were also capable of potently suppressing the ATP-induced release of TNF (by 57 and 60%, respectively). Next we examined the effects of three of these inhibitors (U0126, SP600125, and SB203580) on the intracellular accumulation of TNF in microglia during a 2 hr period of stimulation with ATP to determine whether all three MAP kinases are involved in the regulation of the *de novo* production of TNF. Once again, all three inhibitors markedly reduced the intracellular levels of TNF in ATP-stimulated microglia (Fig. 1*B*). These data suggest that ERK, JNK, and p38 MAP kinase are all involved in both the production and the release of TNF by ATP-treated microglia.

To determine whether all three kinases also regulate TNF transcription, we used real-time RT-PCR analysis to examine the effects of the three inhibitors on ATP-induced TNF mRNA expression. As shown in Figure 1*C*, ATP treatment led to a marked increase in TNF mRNA levels; moreover, the extent of this increase was greatly reduced in the presence of either U0126 or SP600125 but did not appear to be affected by SB203580. Thus, although all three kinases appear to be critical for ATP-induced TNF production, they appear to have different modes of action, with ERK and JNK acting to regulate transcription of the TNF

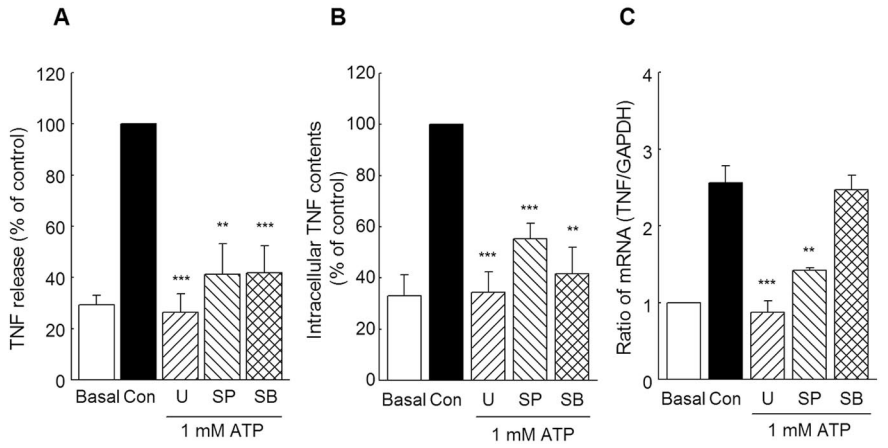

Figure 1. Effects of U0126 (U), SP600125 (SP), and SB203580 (SB) on ATP-induced TNF release, intracellular TNF production, and mRNA expression in microglia. The cells were treated with 10 μM U0126, 30 μM SP600125, or 15 μM SB203580 for 15 min and stimulated with 1 mM ATP for 3 hr (*A*), 2 hr (*B*), and 1 hr (*C*). The released TNF (*A*) and the intracellular TNF contents (*B*) were measured by ELISA. Values are expressed as mean ± SEM of percentage of release compared with ATP alone from three independent experiments. Values of 100% for the release and intracellular production of TNF in ATP-stimulated microglia were 178.8 ± 22.2 and 407.0 ± 45.6 pg/10^6 cells, respectively. *C*, The expression of TNF mRNA was quantified by real-time RT-PCR. Values are shown as the ratio of TNF mRNA versus GAPDH mRNA. Data are expressed as mean ± SEM of ratio of expression compared with ATP or BzATP alone from three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$, significantly different from the control (Con) (*t* test).

gene and p38 only acting post-transcriptionally to interfere with the production or release of TNF.

p38 mediates the transport of TNF mRNA from the nucleus to the cytoplasm

A recent report indicates that the nucleocytoplasmic transport of TNF mRNA in lipopolysaccharide (LPS)-stimulated macrophages is regulated by ERK-dependent signals (Dumitru et al., 2000). We therefore attempted to clarify the mechanism by which p38 mediates post-transcriptional regulation of TNF production by examining the localization of TNF mRNA in the nucleus and the cytoplasm of microglia. We used Western blots of the marker proteins octamer-binding protein 1 (Oct-1) and heat shock protein 90 (Hsp-90) to confirm the efficacy of the technique used to separate the nuclear and cytoplasmic fractions (Fig. 2*B*). Our results indicated that ATP treatment led to significantly increased TNF mRNA levels in the cytoplasm as well as in the nucleus; presumably, this meant that the TNF mRNA had been exported from the nucleus to the cytoplasm so that new TNF protein molecules could then be synthesized on ribosomes (Fig. 2*A*). Interestingly, exposure of ATP-treated microglia to SB203580 did not appear to have any effect on the significantly increased levels of TNF mRNA that we observed in nuclei, but it did appear to prevent any comparable increase in cytoplasmic TNF mRNA levels from becoming evident (Fig. 2*A*). These data could be indicating that p38 has a role in regulating the nucleocytoplasmic transport of TNF mRNA in ATP-stimulated microglia, although we cannot rule out other possible explanations, including, for example, differences in the half-life of TNF mRNA in the nucleus and the cytoplasm.

P2X$_7$ receptors are coupled to JNK and p38 activation but not to ERK activation

One reason for suspecting that ATP-induced TNF release is mediated by P2X$_7$ receptors is that the optimal concentration of ATP that induces TNF release is approximately equivalent to the concentration needed to activate P2X$_7$ (i.e., of the order of 1 mM). Moreover, although we reported previously that BzATP (a P2X$_7$ agonist) is able to mimic the effects of ATP in provoking TNF release (Hide et al., 2000), we now find that α,β-methylene ATP (which is known to be a P2X receptor agonist and not a P2X$_7$ agonist) has no such effect (data not shown). We were able to confirm the involvement of P2X$_7$ receptors by showing that brilliant blue G (BBG), a P2X$_7$ receptor blocker, suppresses ATP- as well as BzATP-induced TNF release (Fig. 3*A*). We then tried to determine whether P2X$_7$ receptors are able to activate MAP kinases by examining the effects of BBG on the ATP-induced activation of ERK, JNK, and p38. The results in Figure 3*C* indicate that BBG is capable of selectively inhibiting the ATP-induced activation of JNK and p38 but appears to have little if any effect on the activation of ERK. Similar results were obtained using BzATP-stimulated cells (Fig. 3*B,D*). These results indicate that the activation of P2X$_7$ receptors does lead to JNK and p38 activation, and that ERK activation occurs by some other, as yet unidentified, route.

The P2X$_7$ receptor-mediated activation of JNK and p38 is protein tyrosine kinase dependent

Despite the fact that the P2X$_7$ receptor can and does function as a cation channel, activation of JNK and p38 appears to be independent of extracellular Ca^{2+} (Hide et al., 2000) and may therefore be dependent on a mechanism that is independent of Ca^{2+} influx. We therefore decided to investigate a possible involvement of PTK (protein tyrosine kinase) in this pathway by studying the effects of a general PTK inhibitor, genistein, on both TNF release and the activation of each of the MAP kinases that are induced by ATP. We found that genistein significantly inhibited the ATP-induced activation of JNK and p38 (but not that of ERK) (Fig. 4*C*) as well as the ATP-induced release of TNF, and that it did all of these things in a concentration-dependent manner (Fig. 4*A*). We obtained similar results with a second PTK inhibitor known as tyrphostin A25 (data not shown). We then found that the *src*-family PTK inhibitor PP2 also appeared to suppress ATP-induced TNF release, and that its negative control counterpart PP3 had no comparable effect (Fig. 4*B*). PP2 also appeared to inhibit the BzATP-induced activation of JNK and p38 but not that of ERK (Fig. 4*D*). Together, these findings suggest that a PTK, almost certainly a *src*-family PTK, can act downstream of the P2X$_7$ receptor to activate both JNK and p38; this could then lead to the synthesis and subsequent transport of TNF mRNA.

P2X$_7$ receptor-stimulated microglia rescue the neurons from glutamate-induced cell death by releasing TNF

Possible interactions between microglia, the TNF they produce, and neuronal tissue are still not well understood. To determine whether P2X$_7$ receptor-stimulated microglia and the TNF they release are harmful or protective to surrounding neurons, we developed a neuron–microglia coculture system in which primary cultured neurons and microglia are held in separate compartments that nonetheless allow them to exchange freely diffusible factors. A preliminary experiment then showed that even a

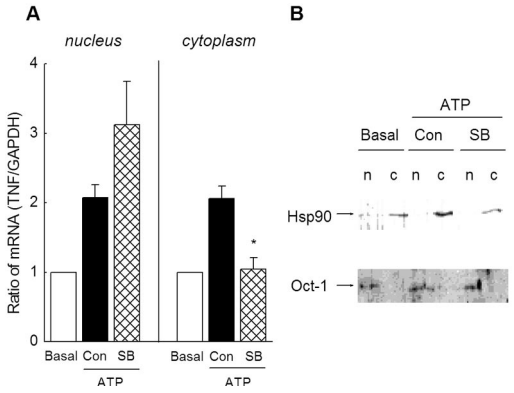

Figure 2. Distribution of TNF mRNA induced by ATP in the nucleus and cytoplasm of microglia treated with SB203580 (SB). *A*, The cells were treated with 15 μM SB203580 for 15 min and stimulated with 1 mM ATP for 1 hr. Nuclear and cytoplasmic fractions of the cells were separated by NE-PER kit, and RNA was extracted from each fraction. Values are shown as the ratio of TNF mRNA levels versus GAPDH mRNA levels. Data are expressed as mean ± SEM of ratio of expression compared with ATP alone from three independent experiments. *$p < 0.05$ significantly different from the control (Con) (*t* test). *B*, Nuclear and cytoplasmic extracts were separated by SDS-PAGE and probed with antibodies to Oct-1 [nuclear protein (n)] and Hsp-90 [cytoplasmic protein (c)] to determine the efficiency of nucleocytoplasmic separation.

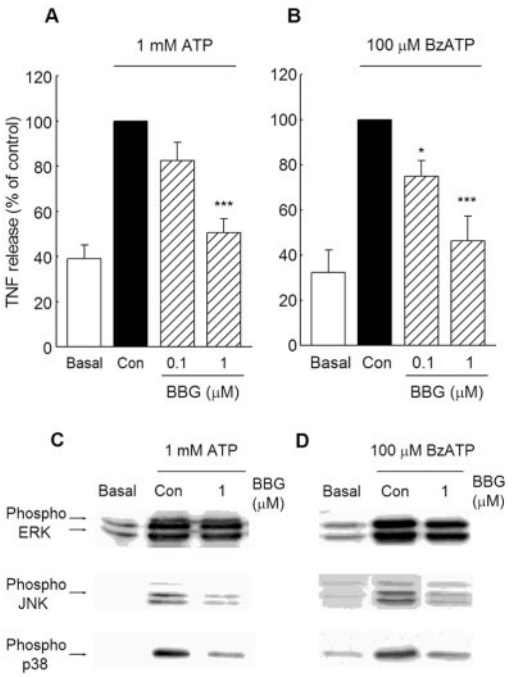

Figure 3. Effects of BBG on the release of TNF and the activation of ERK, JNK, and p38 MAP kinase in ATP- or BzATP-stimulated microglia. The cells were treated with BBG (0.1 or 1 μM) for 5 min and stimulated with 1 mM ATP (*A*, *C*) or 100 μM BzATP (*B*, *D*) for 3 hr (*A*, *B*) or 10 min (*C*, *D*). *A*, *B*, The release of TNF was measured by ELISA. Values are expressed as mean ± SEM of percentage of release compared with ATP or BzATP alone from six independent experiments. Values for 100% of release of TNF were 183.8 ± 46.3 and 350.7 ± 152.2 pg/10^6 cells in ATP- or BzATP-stimulated microglia, respectively. *$p < 0.05$; ***$p < 0.001$, significantly different from the control (Con) (*t* test). *C*, *D*, The phosphorylated (active) and total ERK, JNK, and p38 were detected by Western blotting using antibodies that recognize phosphorylated and both phosphorylated and nonphosphorylated enzymes, respectively. The levels of each total MAPK were confirmed to be identical for each lane. Similar results were obtained in at least three independent experiments.

brief (10 min) exposure of neurons to 100 μM glutamate followed by culture for 24 hr led to ~50% of them dying (Fig. 5*A*). There was no significant difference in glutamate neurotoxicity between cultures of neurons alone and neuron–microglia cocultures (Fig. 5*A*,*B*), although the neurons in cocultures that had been exposed to BzATP for 24 hr before the addition of glutamate appeared to have been given a significant level of protection against glutamate-induced cell death (Fig. 5*B*). No comparable protection was evident in neurons that were cultured on their own with BzATP (Fig. 5*A*), nor was there any evidence of a reduction in their viability (Fig. 5*A*,*B*). Interestingly, we also found that TNF-α protease inhibitor (TAPI), a well known inhibitor of TNF-α-converting enzyme (TACE), strongly inhibited the BzATP-induced release of TNF (Fig. 5*D*), and that both TACE and anti-TNF antibody significantly suppressed the protective effect that is afforded to neurons by BzATP-treated microglia (Fig. 5*C*). Furthermore, this protective effect was abolished by BBG, confirming the involvement of $P2X_7$ receptor (Fig. 5*C*). Similar results were obtained using ATP-stimulated microglia (data not shown). Together, our results strongly indicate that the neuroprotective effect of $P2X_7$ receptor-stimulated microglia stems very directly from their ability to release TNF. Whether other substances are involved remains to be seen.

Discussion

In the present study, we have shown that three MAP kinase family members (ERK, JNK, and p38) all make important contributions to the ATP-induced production of TNF in microglia, and that their precise individual roles may differ. Thus, ERK and JNK appear to regulate TNF mRNA synthesis, whereas the effects of p38 seem to be limited to the post-transcriptional level and probably involve the nucleocytoplasmic transport of TNF mRNA. Other findings led us to suggest that activation of JNK and p38 by the $P2X_7$ receptor is likely to be mediated by a *src*-family PTK. This study also revealed the biological significance of the TNF released from $P2X_7$ receptor-activated microglia.

The precise mode of action of ERK and JNK in microglial TNF production is not yet known, but it may be that they exert their effects on the promoter domain of the TNF gene by activating one of the nuclear factor-κB, nuclear factor of activated T cells, or activator protein-1 transcription factors. As the p38-mediated post-transcriptional regulation of TNF production, it is known, for example, that p38 regulates the expression of several RNA-binding proteins, which then help to stabilize TNF mRNA by interacting with the adenine uracil-rich element (ARE) region in the 3′ untranslated region of TNF mRNA (Kontoyiannis et al., 2001; Mahtani et al., 2001). Other proteins such as eukaryotic polypeptide chain initiation factor-4E, eukaryotic polypeptide chain elongation factor-1, and eukaryotic polypeptide chain releasing factor-1 also appear to contribute to translational efficiency, whereas elF-4E, a translation start factor, responds to a regulatory process downstream of p38 MAP kinase (Lee et al., 2000). Interestingly, Dumitru et al. (2000) have suggested that the transport efficacy of TNF mRNA is regulated through the ARE via ERK, although our findings suggest that p38-mediated post-transcriptional regulation of TNF production regulates the nucleocytoplasmic transport of TNF mRNA in ATP-activated microglia. Another recent paper suggested that MAP kinase-activated protein kinase 2 (MAPKAPK2), which is activated in response to direct phosphorylation by p38, also appears to be involved in the post-transcriptional regulation of TNF biosynthesis in LPS-stimulated macrophages, but it is not yet known whether MAPKAPK2 participates directly in the regulatory process (Kotlyarov et al., 1999).

Recent reports indicate that $P2X_7$ receptors are implicated in diverse cell functions, such as apoptosis, transcription, microvesicle shedding, and synaptic transmission (Humphreys et

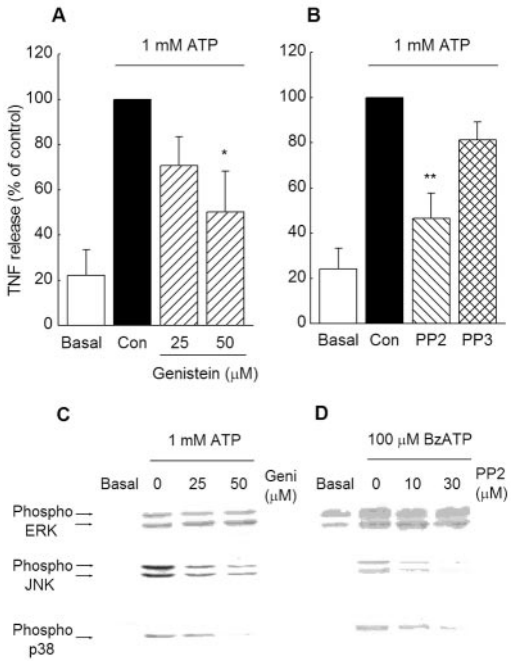

Figure 4. Effects of genistein and PP2 on the release of TNF and the activation of ERK, JNK, and p38 MAP kinase induced by ATP. Microglia were treated with genistein, a nonselective PTK inhibitor (*A, C*), with PP2, an *src*-family-selective PTK inhibitor, or with PP3, an inactive analog of PP2 (*B, D*); stimulated with 1 mM ATP or 100 μM BzATP; and then measured for the release of TNF after 3 hr (*A, B*) and MAP kinase activation after 10 min (*C, D*). Values are expressed as mean ± SEM of percentage of release compared with ATP alone from three independent experiments. Values of 100% for the release of TNF were 136.0 ± 37.0 (*A*) and 133.6 ± 65.1 (*B*) pg/10^6 cells. $*p < 0.05$; $**p < 0.01$, significantly different from the control (Con) (*t* test). *C, D*, The phosphorylated (Phospho) (active) and total ERK, JNK, and p38 were detected by Western blotting using antibodies that recognize either phosphorylated or both phosphorylated and unphosphorylated enzymes, respectively. The (total) levels of each MAP kinase were confirmed to be identical for each lane. Similar results were obtained in at least three independent experiments. Geni, Genistein.

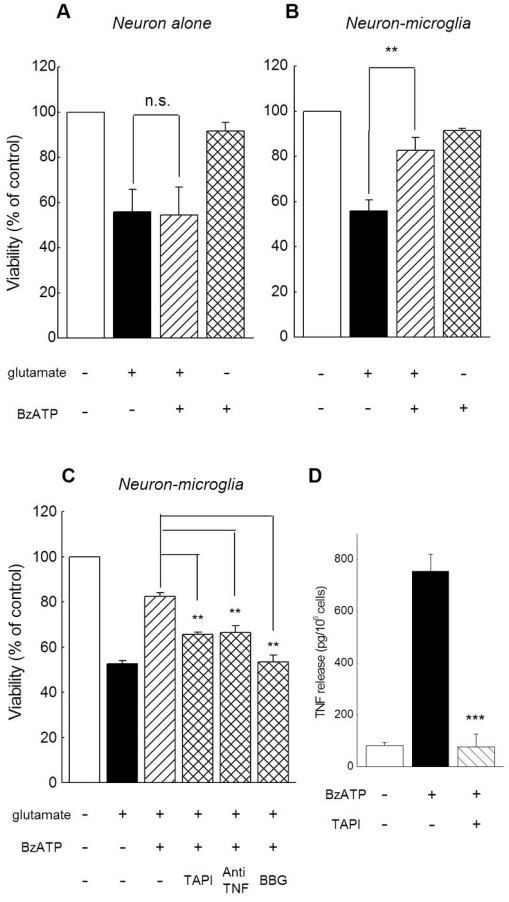

Figure 5. Effects of BzATP on the viability of primary cortical neurons in "neuron alone" cultures or neuron–microglia cocultures. Primary cultures of rat cortical neurons alone (*A*) or cocultures of neurons with microglia (*B*) were treated with 100 μM BzATP for 24 hr and then stimulated with 100 μM glutamate for 10 min. After 24 hr of incubation, neuronal cell viability was determined by MTT assay. Values are expressed as mean ± SEM of percentage of viability of control cells from three independent experiments. $**p < 0.01$, significantly different from glutamate alone (*t* test). n.s., Not significant. *C*, Effects of TAPI, TACE inhibitor, anti-TNF, and BBG on BzATP-induced neuroprotection. Cortical neurons cocultured with microglia were treated with 50 μM TAPI, 10 μg/ml anti-TNF, and 1 μM BBG for 5 min before BzATP application. After 24 hr of coculture, the neurons were stimulated with 100 μM glutamate for 10 min. After 24 hr of additional incubation, neuronal cell viability was determined by MTT assay. Values are expressed as mean ± SEM of percentage of viability of control cells from three independent experiments. $**p < 0.01$, significantly different from BzATP– glutamate application (*t* test). *D*, Effects of TAPI on BzATP-induced TNF release from rat microglia. $***p < 0.001$.

al., 2000; MacKenzie et al., 2001; Armstrong et al., 2002), and that they have a role in the release of cytokines such as IL-1β and IL-18 (Sanz and Di Virgilio, 2000; Mehta et al., 2001). Activation of $P2X_7$ receptors may also be involved in triggering the release of TNF from rat microglia, given that: (1) a relatively high concentration of ATP (1 mM) is needed to ensure that TNF is released, (2) the $P2X_7$ agonist BzATP is much more effective as an inducer of TNF release than ATP (Hide et al., 2000), and (3) the $P2X_7$ antagonist BBG is a powerful inhibitor of ATP–BzATP-induced TNF release (Fig. 3*A*,*B*). We have also shown that BBG inhibits the activation of JNK and p38 but not ERK, thereby indicating that $P2X_7$ receptors may well be involved in the activation of JNK and p38. The $P2X_7$ receptor signaling pathways that lead up to the activation of MAP kinases still have to be properly characterized. Our data suggest that a PTK may be involved in activating JNK and p38 but not ERK. Although we do not know which PTK is involved, it may be an *src*-family tyrosine kinase such as *Lyn* or *Lck*, primarily because the *src*-family PTK-selective inhibitor PP2, but not its negative control PP3, strongly suppresses JNK and p38 activation as well as ATP-induced TNF release. As suggested recently by Denlinger et al. (2001), the Src homology 3 (SH3) domains of *src*-family kinases may well interact with the SH3 domain-binding motif in the $P2X_7$ C-terminal domain. It has also been reported that AMPA receptors, which also act as glutamate ion channel receptors, are capable of promoting glial cell line-derived neurotrophic factor release by a mechanism that is: (1) dependent on the *Lyn* tyrosine kinase and (2) independent of ion flux (Hayashi et al., 1999). ATP-induced p38 and JNK activation does not appear to be associated with Ca^{2+} influx (Hide et al., 2000; our unpublished observation), so it is conceivable that any *src*-family PTK family that plays a part in the transduction of the $P2X_7$ receptor does so because of its C-terminal domain rather than because of its ability to function as an ion channel.

$P2X_7$ receptors are believed to be involved in the activation of ERK in several cell types (Panenka et al., 2001; Budagian et al., 2003; Gendron et al., 2003). In the present study, however, activation of ERK by ATP (or even by the more powerful BzATP) was not sensitive to the $P2X_7$ antagonist BBG. Several lines of evidence point to the involvement of other P2 receptors in the activation of ERK. It has been demonstrated, for instance, that in rat primary astrocytes, ATP activates ERK via a P2Y–PKCδ–phospholipase D pathway (Neary et al., 1999), whereas the ATP re-

leased during stretch is more likely to activate ERK through $P2X_2$ and $P2Y_1$ (Neary et al., 2003). ADP and UTP also activate ERK possibly via $P2Y_{12}$ and $P2Y_2$ receptors expressed on microglia. Together, these findings lend support to the view that ERK activation may involve several non-$P2X_7$ P2 receptors. However, a recent study in which $P2X_7$ C-terminal and N-terminal mutants were expressed in human embryonic kidney 293 cells established that the N terminal of $P2X_7$ has an essential role in ERK activation and that the C terminal is required for Ca^{2+} entry (Amstrup and Novak, 2003). One possible explanation for the obvious discrepancy between these results and our findings could be that the $P2X_7$ N-terminal-associated activation signals are insensitive to BBG; unfortunately, we do not yet know whether this is the case. Nevertheless, ERK activation is clearly important for TNF-producing cells, given that it plays a key role in both the production of TNF and its subsequent release and that it does so by modulating the activity of TACE, a protein that converts 26 kDa membrane-bound pro-TNF into 17 kDa soluble TNF at the cell surface (Bezzi et al., 2001).

It is still not entirely clear whether microglia protect or harm neurons or whether TNF is beneficial or toxic (Arnett et al., 2001; Combs et al., 2001; Fontaine et al., 2002). Thus, for example, TNF appears to enhance injury, as shown by the fact that the injection of neutralizing TNF antibody into lesion sites significantly reduces experimental ischemic and traumatic injury (Barone et al., 1997; Meistrell et al., 1997). There is also recent evidence that indicates that TNF can provide protection to neurons because it is able to encourage the expression of anti-apoptotic and anti-oxidative proteins. Moreover, evidence from experiments in TNF-deficient mice indicates that although TNF has a deleterious effect during the acute response that occurs in a traumatized brain, it also has a key part to play in both the long-term behavioral recovery and the histological repair of the tissues (Scherbel et al., 1999). It is of course possible that the effects of TNF depend just as much on the postinjury time course as on its expression levels. Recent reports indicate that the dual actions of TNF are mediated via different TNF receptors, with the p55 TNF receptor 1 (TNFR1) eliciting neurotoxic effects and the p75 TNF receptor 2 (TNFR2) eliciting neuroprotection (Yang et al., 2002). Interestingly, these two receptors have been shown to have similarly specific roles in oligodendrocytes, with TNFR1 being implicated in demyelination and TNFR2 in remyelination (Arnett et al., 2001).

Given that one of our primary aims in this study was to better understand the biological significance of the TNF that is released from ATP- or BzATP-activated microglia, we decided to construct a primary neuron–microglia coculture system that could be used to screen for possible effects. Our results clearly demonstrate that BzATP-stimulated microglia provide neurons with effective protection against glutamate-induced cell death. This protective effect appears to be mediated by a soluble factor or factors released from $P2X_7$-activated microglia. One such neuroprotective factor is almost certain to be TNF, because microglia-mediated neuroprotection was suppressed in the presence of either TACE inhibitor or anti-TNF antibody. Given that ATP is likely to be released by cells that have been damaged by trauma, inflammation, or ischemia, and that low ATP concentrations can lead to chemotaxis of microglia (Honda et al., 2001), it seems not unreasonable to hypothesize that ATP can act as an emergency messenger that recruits microglia to a damaged brain area, at which point a pathway that involves ATP-activated $P2X_7$ receptors takes over and provokes the microglia to secrete neuroprotective factors such as TNF.

Here, we provided new information about the mechanisms that underlie the production of TNF via $P2X_7$ receptors and MAP kinases in rat microglia and have clearly established a neuroprotective role for the TNF that the ATP-activated microglia may go on to release. A better understanding of the mechanisms by which microglia are transformed into cells that can protect neurons, including the mechanism that leads to $P2X_7$ receptor activation, will undoubtedly help in the development of more rational approaches to the entire spectrum of neural diseases.

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