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Release of Calcium from Inositol 1,4,5-Trisphosphate Receptor-Regulated Stores by HIV-1 Tat Regulates TNF-α Production in Human Macrophages

Michael Mayne,* Clark P. Holden,* Avindra Nath,† and Jonathan D. Geiger2*

HIV-1 protein Tat is neurotoxic and increases macrophage and microglia production of TNF-α, a cytopathic cytokine linked to the neuropathogenesis of HIV dementia. Others have shown that intracellular calcium regulates TNF-α production in macrophages, and we have shown that Tat releases calcium from inositol 1,4,5-trisphosphate (IP₃) receptor-regulated stores in neurons and astrocytes. Accordingly, we tested the hypothesis that Tat-induced TNF-α production was dependent on the release of intracellular calcium from IP₃-regulated calcium stores in primary macrophages. We found that Tat transiently and dose-dependently increased levels of intracellular calcium and that this increase was blocked by xestospongin C, pertussis toxin, and by phospholipase C and type 1 protein kinase C inhibitors but not by protein kinase A or phospholipase A₂ inhibitors. Xestospongin C, BAPTA-AM, U73122, and bisindolylmaleimide significantly inhibited Tat-induced TNF-α production. These results demonstrate that in macrophages, Tat-induced release of calcium from IP₃-sensitive intracellular stores and activation of unconventional PKC isoforms play an important role in Tat-induced TNF-α production. The Journal of Immunology, 2000, 164: 6538–6542.

Patients with HIV-1 dementia suffer multiple cognitive and behavioral deficits and usually only survive a few months following onset (1). Despite identifying characteristic clinical symptoms of HIV dementia, little is known about the molecular mechanisms that regulate its development and progression. Macrophages and microglia are productively infected with HIV-1 (2–4) and shed intact HIV virions and viral proteins (including Tat) that can act directly on adjacent cells to cause increased levels of intracellular calcium ([Ca²⁺]i) (5), neurotoxicity (6–8), and subsequent neurodegeneration (9).

The HIV-1 protein Tat may be particularly important in HIV-associated neurodegeneration because of the following findings. HIV-1 Tat protein (9) and transcripts (10) are present in autopsy brain samples from HIV-infected patients with dementia. Tat is released from HIV-infected cells (11, 12). Primary neurons and astrocytes exposed to Tat rapidly release calcium from inositol 1,4,5-trisphosphate (IP₃)-regulated pools and, subsequent to this release of calcium, extracellular calcium that enters the cell leads to calcium dysregulation and neuron cell death (5, 9, 13). Tat activates primary astrocytes, peripheral blood macrophages, and microglia to produce proinflammatory cytokines including IL-1, IL-6, and TNF-α (14–16), and even a transient exposure of monocyte and glial cells to Tat increases cytokine production (15, 17). A neutralizing Ab to TNF-α blocks Tat-induced neurotoxicity (8). Together, these results strongly suggest that Tat protein can activate calcium mobilization in multiple cell types within the brain and concurrently induce proinflammatory cytokine production.

We reported recently that Tat induces IP₃-regulated calcium release in neurons and astrocytes and that this increase leads to a dysregulation in [Ca²⁺]i and neurotoxicity (5). Others have shown that intracellular stores of calcium play important roles in regulating TNF-α production in primary human macrophages (18–22). Because TNF-α has been implicated as a pathogenic factor in HIV disease (23), is elevated in the brains of HIV-infected patients (24), and Tat has been shown to elevate TNF-α production in monocytes (14, 25), these events may lead to highly activated microglia and macrophages in the brain, an event that correlates with the clinical symptoms of AIDS dementia (3). Thus, it is important to identify the cellular mechanisms that mediate these pathways. Accordingly, we tested the hypothesis that Tat-induced TNF-α production involved the release of [Ca²⁺]i from IP₃-regulated calcium stores in primary macrophages. Here, we report that exposure of human primary macrophages to Tat protein induces a rapid and dose-dependent release of calcium from IP₃-regulated intracellular stores and that Tat-induced TNF-α production was dependent, at least in part, on the release of calcium from those stores.

Materials and Methods

Chemicals and recombinant Tat

ATP, caffeine, [1-(5-isoquinolinesulfonyl)-2-methylpyrazine] hydrochloride (H7), [N-2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide hydrochloride (H89), EDTA, EGTA, pertussis toxin (PT), cholaera toxin (CT), citoilnine (CIT), and 4-bromophenyl bromide (BPB) were purchased from Sigma (St. Louis, MO). Xestospongine C (X,C) and bisindolylmaleimide...
(Bis) were purchased from Calbiochem (San Diego, CA). Fura-2-acetoxyethyl ester (fura-2-AM) and bis(2-aminophenox)ethane-N,N,N',N'-tetracetate acetoxyethyl ester (BAPTA-AM) were obtained from Molecular Probes (Eugene, OR). The phospholipase C (PLC) inhibitor U73122 was purchased from Research Biochemicals (Natick, MA). Anti-ferretin Abs were purchased from Transduction Laboratories (Lexington, KY). Tat-a2 was prepared and purified as described previously (26) and its biological activity was confirmed by activation of β-galactosidase in transfected HeLa cells (AIDS Repository, National Institutes of Health). Tat protein was lyophilized and stored at -80°C. Freshly thawed Tat was used in all experiments.

Preparation of primary macrophage cultures

Human PBMC and macrophages were purified from whole blood obtained from healthy volunteers (27), and cells were cultured at a density of 2.0 × 10^6 cells/ml for 7 days in RPMI 1640 supplemented with 10% FBS and antibiotics. All cells were maintained at 37°C in a humidified growth chamber supplemented with 5% CO₂.

Levels of [Ca²⁺]i

[Ca²⁺], were determined using the Ca²⁺-specific fluorescent probe fura 2-AM as described previously (28). Macrophages were excited at 340 and 380 nm, and emission was recorded at 510 nm with a video-based universal imaging system (EMPIX, Mississauga, ON). R_max/R_min ratios were converted to nanomolar [Ca²⁺], (29), and pressure application of Tat and image acquisition were performed as described previously (5). Peak increases of [Ca²⁺], were determined by subtracting baseline [Ca²⁺], from the maximum [Ca²⁺], achieved during a 15-min period following Tat application.

Macrophage TNF-α production following Tat application

Primary macrophages were treated with Tat (100 nM) and incubated for 4 h at 37°C. Supernatants were collected, centrifuged at 700 × g for 5 min, and analyzed for TNF-α by ELISA (30). For positive controls, cells were treated with 10 ng/ml LPS (LPS, Escherichia coli type 055:B5; Sigma) for 4 h. In experiments where antagonists or inhibitors were used, these agents were added 30 min before stimulation with Tat. Following a 4-h incubation, cell culture supernatants were collected and assayed for TNF-α abundance by ELISA.

Statistical analysis

Significant differences between groups were determined by one-way ANOVA with Tukey’s posthoc comparisons. For all tests, statistical significance was considered to be at the p < 0.01 level (Instat2; Graphpad Software, San Diego, CA).

Results

Dose-related effects of HIV-1 Tat on macrophage [Ca²⁺],

We reported previously that Tat application to cultured human astrocytes and neurons increased [Ca²⁺], (5). We found here that Tat (100 nM) applied to highly purified macrophage cultures (>97% of the cells in the primary culture were immunoreactive with anti-ferretin Ab; data not shown) increased significantly (p < 0.01) [Ca²⁺], from basal levels of 129 ± 1 nM (n = 718; data not shown) to maximum levels of 793 ± 69 nM (n = 42) (Fig. 1). These increases in [Ca²⁺], were dose related with an apparent EC₅₀ value of 6.0 ± 0.3 nM. Independent of the experimental conditions outlined below, agonist or antagonist treatments did not reduce cell viability as determined by trypan blue exclusion (data not shown).

Tat releases calcium from IP₃-regulated stores via a PT-sensitive PLC-mediated pathway

In our previous work with cultured human neurons and astrocytes, we reported that Tat-induced initial transient increases in [Ca²⁺], were due to release from IP₃-regulated stores (5). Similarly, in primary macrophages, Tat induced a sharp and transient increase in [Ca²⁺], (Fig. 2A). To determine the source of this release, we first used the specific inhibitor of IP₃-dependent calcium release, X,C, and found that it significantly reduced (p < 0.01) Tat-induced calcium transients by 91 ± 2% (Fig. 2B; n = 20). As a positive control, X,C significantly blocked (p < 0.001) ATP-induced release of [Ca²⁺], by 90 ± 7% (Fig. 2B; n = 28); ATP is a well-characterized releaser of calcium from IP₃ receptor-regulated stores. Caffeine (20 mM; n = 18) did not increase [Ca²⁺], and ryanodine (10 μM; n = 25) did not affect Tat-induced increases in [Ca²⁺], (data not shown). These latter results indicate that calcium in these cells was not being released from caffeine-sensitive ryanodine receptor-regulated intracellular stores. When Tat was applied to macrophages in calcium-free buffer, increases in [Ca²⁺], were not significantly different from increases observed in cells bathed in media containing calcium (Fig. 2B).

To determine signaling events mediating Tat-induced release of calcium from IP₃-regulated stores, we used selective inhibitors that target potential activators and effectors of PLC. PT (100 ng/ml) significantly decreased (p < 0.001) Tat-induced increases in [Ca²⁺], by 95 ± 1% (Fig. 2C; n = 50). CT (100 ng/ml) did not reduce Tat-induced increases in [Ca²⁺], (Fig. 2C; n = 44). The PLC inhibitor U73122 significantly reduced (p < 0.01) Tat-induced increases in [Ca²⁺], by 90 ± 3% (n = 23). Because phospholipase A₂ (PLA₂) inhibition has been shown to be dependent on PLC activity (31, 32), we tested the involvement of PLA₂ in Tat-induced increases in [Ca²⁺]. The selective PLA₂ inhibitor citicoline (10 μM) did not inhibit Tat-induced increases in [Ca²⁺], (Fig. 2C; n = 9). However, inhibition of protein kinase C (PKC) type 1 isoforms using H7 or Bis significantly decreased (p < 0.01) Tat-induced increases in [Ca²⁺], by 70 ± 8% (n = 59) and 76 ± 11% (n = 41), respectively (Fig. 2C). Inhibition of protein kinase A (PKA) using H89 (n = 27) did not attenuate Tat-induced increases in [Ca²⁺], (Fig. 2C).

Tat-induced increases in TNF-α involved rapid calcium release from IP₃-sensitive stores

Because TNF-α production has been shown to be dependent on increases in [Ca²⁺], (18–22), we hypothesized that transient increases in [Ca²⁺], induced by Tat may increase the levels of Tat-induced TNF-α production (14, 25). LPS and Tat significantly increased (p < 0.001) the levels of TNF-α (Fig. 3A). When LPS or Tat was applied under calcium-free conditions, TNF-α production was inhibited significantly (p < 0.01) by 72 ± 6% and 78 ± 11%, respectively, compared with LPS or Tat applied under calcium-containing conditions (Fig. 3A). In the absence of extracellular calcium, Tat-induced increases in TNF-α were significantly inhibited (p < 0.001) with BAPTA-AM by 86 ± 5% and with X,C by 64 ± 7% (Fig. 3A). In the presence of calcium, neither
BAPTA-AM (1 or 10 μM) nor XsC (1 or 10 μM) significantly inhibited TNF-α production (data not shown). We showed previously in monocytes that U73122 inhibited Tat-induced TNF-α production by 80% (14). In confirmation, in primary macrophages, U73122 significantly inhibited (p < 0.001) Tat-induced increases in TNF-α by 95 ± 2% (Fig. 3B). In attempting to identify G proteins involved in this PLC-mediated response, we conducted the following experiments and found that PT, an inhibitor of G proteins, CT, an activator of Gαi proteins, and BPB or CIT, specific inhibitors of PLA2, did not inhibit Tat-induced TNF-α production (Fig. 3B). Because inhibition of PLC blocked
Tat-induced calcium release from IP$_3$ receptor-regulated stores and TNF-α production, we determined the extent to which PKC-mediated Tat-induced TNF-α production. In agreement with our previous work in THP-1 cells (14), H7 (an inhibitor of PKC type 1 isoforms) did not inhibit Tat-induced TNF-α production (Fig. 3C). Bis, an inhibitor of PKC type 1 isoforms at a low concentration of 50 nM (33), did not reduce TNF-α production in primary macrophages exposed to 100 nM Tat (Fig. 3C). However, at a higher concentration of 6 μM, Bis significantly inhibited ($p < 0.01$) Tat-induced increases in TNF-α production by 53 ± 8% suggesting that nonconventional PKC isoforms mediated Tat-induced increases in TNF-α production (34).

**Discussion**

Apoptotic and/or necrotic neuronal cell death associated with HIV dementia appears to be caused by indirect mechanisms induced by HIV proteins including increases in levels of [Ca$^{2+}$]i, (5) and proinflammatory cytokines (14, 15). The HIV-1 protein Tat may be a particularly important pathogenic factor in the development and progression of dementia because Tat has been shown to increase levels of [Ca$^{2+}$]i (5, 35), increase neuronal cell death (35, 36), and increase TNF-α, IL-1β, IL-1α, and IL-6 production (8, 14, 15). Here, we focused our experiments to determine signaling events through which Tat drives TNF-α production. This issue is important because Tat protein is present in the brain of patients with HIV dementia (36–38). Tat transcripts are elevated in brains of patients with HIV-1 dementia and encephalitis (38), and primary macrophages, which can be recruited into the CNS upon activation and from which proinflammatory cytokines are primarily released, are activated by HIV-1 Tat (14, 15, 37).

Similar to our previous findings in neurons and astrocytes (5), we found, in macrophages, that Tat caused rapid and transient increases in [Ca$^{2+}$]i, even in the absence of extracellular calcium. These findings suggest that Tat-induced increases in [Ca$^{2+}$]i originated from [Ca$^{2+}$]i stores. The calcium released by Tat originated from IP$_3$-regulated pools in a PT-sensitive PLC-mediated manner. This conclusion is based on our observations that Tat-induced increases in [Ca$^{2+}$]i were inhibited significantly, by X C, a selective inhibitor of IP$_3$ release channels regulated by IP$_3$ receptors, by PT, an inhibitor of G$_i$ proteins, and by U73122, a PLC inhibitor. Tat-induced increases in [Ca$^{2+}$]i were not due to release of [Ca$^{2+}$]i from ryanodine receptor-regulated [Ca$^{2+}$]i release channels nor the endoplasmic reticulum-resident calcium release channels in addition to those regulated by IP$_3$ receptors, because neither caffeine nor ryanodine affected Tat-induced increases in [Ca$^{2+}$]i. In contrast to our observations in some neurons and astrocytes (5), Tat did not induce sustained increases in [Ca$^{2+}$]i, in any of the macrophages examined. The increase in [Ca$^{2+}$]i, by Tat that originated primarily from intracellular stores was dependent on PKC type 1 and nonconventional PKC isoforms. This latter observation, in combination with our previous results that tyrosine kinases were involved in Tat-induced TNF-α production (14), suggests that Tat activates multiple kinase pathways.

Several studies have demonstrated that in monocytes the release of IP$_3$ receptor-regulated [Ca$^{2+}$]i stores results in increased TNF-α production (18–22, 39). In concert with those findings, we found that Tat-induced increases in calcium release from IP$_3$ receptor-regulated stores are also involved in TNF-α production. Although we did not observe any evidence for extracellular calcium influx in primary macrophages, our results do demonstrate a role for extracellular calcium, in addition to [Ca$^{2+}$]i, in regulating Tat-induced TNF-α production. This is in contrast to some of our previous observations in neurons and astrocytes where we did observe some evidence of extracellular calcium influx (5). The likely explanation for not observing evidence of calcium influx in macrophages, but yet seeing an involvement of extracellular calcium in TNF-α production, is that the rapid and transient release of calcium from IP$_3$ receptor-regulated stores resulted in the activation of capacitative mechanisms and small increases in [Ca$^{2+}$], that were not detectable in our system. Thus, the temporal order of signaling events that occurs in macrophages activated by Tat to produce TNF-α are increases in [Ca$^{2+}$], that precede capacitative entry of extracellular calcium. This conclusion is supported by our observations that U73122, a selective inhibitor of PLC, reduced significantly increases in [Ca$^{2+}$], and TNF-α production even in the presence of extracellular calcium. Our conclusion that calcium release from IP$_3$ receptor-regulated stores initiates Tat-induced production of TNF-α before influx of extracellular calcium is supported further by our findings that the L-type calcium channel inhibitors nimodipine and nicardipine did not significantly reduce Tat-induced increases in TNF-α production (data not shown). Further, it was reported recently that nimodipine did not afford clinical benefit of HIV-1 dementia (40). Thus, in primary macrophages, Tat-induced increases in [Ca$^{2+}$], resulting primarily from release from IP$_3$ receptor-regulated stores may represent the seminal signaling event required for the production of TNF-α.

Although our results demonstrated that Tat activation of PLC is critical for increases in [Ca$^{2+}$], that initiate TNF-α gene expression, additional signaling events are required for maximal TNF-α production. Contrary to our finding that Tat-induced increases in [Ca$^{2+}$], were PT sensitive, TNF-α production was not PT sensitive, suggesting that Tat activated at least two independent G protein signaling pathways. Further, our experiments indicated that Tat-induced TNF-α production involved nonconventional PKC isoforms. This conclusion is based on two observations. First, Bis (6 μM) inhibited both Tat-induced increases in [Ca$^{2+}$], and TNF-α production, and these high concentrations of Bis inhibit nonconventional PKC isoforms (34). Second, although H7, a selective blocker of PKC type 1 isoforms, lowered Tat-induced increases in [Ca$^{2+}$], it did not inhibit TNF-α production. These findings are consistent with findings that Tat activates nonconventional PKC isoforms including ε and ξ in PC12 cells (41), that HIV-1 regulates NF-κB via PKCζ in infected monocytes (42), and that multiple signaling pathways are activated by Tat (9).

Finally, very low concentrations (EC$_{50}$ of 6 nM) of Tat caused significant increases in [Ca$^{2+}$], and TNF-α production in primary macrophages (14). Similar concentrations have been demonstrated in serum from HIV-infected patients (43). It has also been shown that Tat can cross the intact blood-brain barrier (44). Given that even a transient exposure of Tat induces a rapid and sustained production of cytokines (15), even extremely small amounts of Tat present in an HIV-infected brain (37) may efficiently activate infiltrating macrophages and resident microglia to produce excessive amounts of TNF-α. Further, because macrophages and microglia are the most commonly infected cells in AIDS brain (2), Tat release from these cells may lead to persistent activation and excessive production of multiple proinflammatory cytokines and chemokines that are implicated in the development and progression of HIV dementia. We conclude that pharmacological strategies that target the IP$_3$ pathway may be therapeutically beneficial in the treatment of HIV dementia.

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References


