Enhanced Dendritic Cell Antigen Uptake via α2β1 Adrenoceptor-Mediated PI3K Activation Following Brief Exposure to Noradrenaline

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*J Immunol* 2010; 185:5762-5768; Prepublished online 8 October 2010;
doi: 10.4049/jimmunol.1001899
http://www.jimmunol.org/content/185/10/5762
Enhanced Dendritic Cell Antigen Uptake via α₂-Adrenoceptor-Mediated PI3K Activation Following Brief Exposure to Noradrenaline

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Although noradrenaline (NA), a stress-associated neurotransmitter, seems to affect the immune system, the precise mechanisms underlying NA-mediated immunomodulation are not fully understood. We examined the effect of NA on Ag uptake (endocytosis) by dendritic cells (DCs) using murine bone marrow-derived DCs and fluorescence-labeled endocytic tracers (dextran and OVA). Ag uptake by DCs notably increased following a very brief treatment (3 min) with NA. NA-induced endocytosis was completely blocked by treatment with α₂-adrenoceptor antagonist yohimbine. Neither α₁-adrenoceptor antagonist prazosin nor β-adrenoceptor antagonist propranolol affected NA-induced endocytosis by DCs. A selective α₂-adrenoceptor agonist, azepexole (B-HT 933), also significantly increased endocytosis by DCs. Thus, the α₂-adrenoceptor seems to be responsible for NA-induced DC endocytosis. In parallel, NA rapidly enhanced Ag capture by DCs via α₂ adrenoceptor-mediated PI3K activation, which may be associated with immune enhancement following acute stress. *The Journal of Immunology*, 2010, 185: 5762–5768.

Dendritic cells (DCs) are potent APCs and play a major role in the regulation of immune responses to a variety of Ags (7–9). It was reported that NA alters the balance of cytokine production by DCs upon TLR stimulation (10–12). NA treatment for ≥3 h suppresses DC production of proinflammatory cytokines, including IL-12 and TNF-α, whereas it enhances the production of IL-10, an anti-inflammatory cytokine (10–12), similarly to cAMP-elevating agents, such as PGE₂ and forskolin (13). It seems that NA-mediated alteration of cytokine balance is involved, at least in part, in immune suppression upon chronic stress. The AR family is composed of α₁ (α₁A, α₁B, and α₁C), α₂ (α₂A, α₂B, and α₂C), and β (β₁, β₂, and β₃) receptors, coupled with guanine nucleotide-binding protein (G protein) (14–16). The effect of NA on cytokine production seems to be mainly mediated via β ARs coupled with Gs proteins responsible for the elevation of intracellular cAMP levels and the activation of protein kinase A (PKA) (10–12). DCs were shown to express β ARs, as well as α₁ and α₂ ARs. It was reported that α₁ ARs are involved in DC migration to the lymph nodes in mice (17). However, the role of α₂ ARs in DC functions and α₂ AR-mediated signal transduction in DCs remains to be elucidated.

DCs circulate through lymphoid organs, as well as almost all tissues as sentinels in the immune surveillance system (7–9). DCs first capture Ags via endocytosis from extracellular fluid following injury and subsequent invasion of pathogens and then present these Ags in the context of MHC class II molecules at the cell surface to activate the Ag-specific CD4⁺ T cells. In parallel, the sympathetic nervous system seems to release NA in response to injury (18), whereas NA signaling seems to be quickly terminated by diffusion, reuptake, and degradation of this transmitter.

Although DC endocytosis of Ags is essential to induce acquired immunity, the precise mechanisms underlying the regulation of this process are not fully understood. We examined the effect of short-term NA exposure (3–20 min) on the functions of DCs using murine bone marrow-derived DCs (BMDCs). In this article, we demonstrate that NA induces DC endocytosis via α₂ AR signaling within a very short time (3 min). We also demonstrate...
involvement of the PI3K pathway in α2 AR-mediated endocytosis of DCs.

Materials and Methods

Reagents and Abs

RPMI 1640 liquid medium was purchased from Sigma-Aldrich (St. Louis, MO) and supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin.Murine rGM-CSF was obtained from PeproTech (Rocky Hill, NJ). Rabbit complement (Low Tox-M) was acquired from Cedarlane Laboratories (Hornby, Ontario, Canada). Dextran (10,000 m.w.) conjugated with Alexa Fluor 488 (A488-dextran) and OVA conjugated with Alexa Fluor 488 (A488-OVA) were acquired from Molecular Probes (Eugene, OR). Con A conjugated with rhodamine (Rho-ConA) was obtained from Vector Laboratories (Burlingame, CA), NA, prazosin (α1 AR-specific antagonist), yohimbine (α2 AR-specific antagonist), propranolol (β AR-specific antagonist), azepexole (β2 AR-specific antagonist), azepexole (β2 AR-specific antagonist), and a coverslip was placed. Cell morphology and fluorescence intensity following washing, the cells were stained with Rho-ConA. The cells were in a 3–20-min incubation. A488-dextran or A488-OVA was added to a final concentration of 5% FCS. 20 ng/ml GM-CSF, and 50 µM 2- ME at a density of 1×10^6 cells/ml/well using a 24-well plate. On day 2, the medium was gently exchanged with fresh medium. On day 4, nonadherent granulocytes were removed without dislodging clusters of developing DCs, and fresh medium was added. On day 6, free-floating and loosely adherent cells were collected and used as BMDCs (93–97% CD11c+B220^+).

Endocytosis assay

The endocytosis assay was performed as previously described (23, 24). BMDCs (0.5–1×10^6 cells/ml) were incubated in 5% FCS RPMI 1640 buffered with 10 mM HEPES at 37°C. The endocytic tracer (A488-dextran or A488-OVA) and NA were added concurrently, prior to a 3–20-min incubation. A488-dextran or A488-OVA was added to a final concentration of 50 or 100 µg/ml, respectively. Endocytosis of the tracer was halted at the indicated time points by rapid cooling of the cells on ice. The cells were then washed with ice-cold PBS. The fluorescence intensity of the cells was analyzed by flow cytometry on a FACSARia (BD Biosciences, San Jose, CA). Incubation of cells with the endocytic tracer on ice was used as a background control. The mean fluorescence intensity (MFI) resulting from the subtraction of background control from each experimental sample represented the amount of incorporated tracer. To examine the effects of inhibitors on endocytosis, cells were pretreated for 30–60 min with each inhibitor at 37°C. After inhibitor pretreatment, the cells were treated with NA at 37°C in the presence of the inhibitor. Rapid cooling of the cells on ice halted the reactions, and these cells were washed with ice-cold PBS. The whole-cell lysates were prepared using cell lysis buffer (Cell Signaling Technology). The cell lysates were separated by SDS-PAGE and then blotted onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was probed with the primary Ab and developed with HRP-conjugated secondary Ab by ECL.

Cytokine measurement in culture supernatants

BMDCs were treated with LPS and P3C for 24 h in 5% FCS RPMI 1640 at a density of 1×10^6 cells/ml. In some experiments, cells were pretreated with an inhibitor for 60 min and then treated with LPS plus P3C for 24 h in the presence of the inhibitor. The culture supernatants were subjected to quantification of the protein level of IL-12 (p70) and IL-10 by ELISA. The ELISA kits for IL-12 (p70) and IL-10 were purchased from BD Biosciences.

Statistical analysis

The unpaired Student t test was used to analyze data for significant differences; p values <0.05 were regarded as significant.

Results

NA-induced rapid endocytosis in DCs

We examined the effect of NA on DC endocytosis using a fluorescence-labeled dextran, a common endocytic tracer of DCs (23). BMDCs were incubated with A488-dextran in the presence or absence (control) of 1 µM NA for a variable time period, ranging from 3–20 min. Fluorescence intensity of the cells was analyzed by flow cytometry (Fig. 1). In the absence of NA, slight uptake of A488-dextran by DCs was detected 3 min after the tracer addition (Fig. 1, upper panel) and then the fluorescence intensity of the cells was gradually increased at 5–20 min (Fig. 1B). Of note, NA notably increased A488-dextran uptake by DCs at 3 min (Fig. 1A, lower panel), Fig. 1B). Significant enhancement of A488-dextran uptake by DCs was also detected at 5–20 min after the NA treatment (Fig. 1B). Endocytosis of A488-dextran by DCs increased with the addition of NA in a dose-dependent manner, peaking at 1 µM after 3 min of treatment (data not shown).

We next analyzed the morphology of NA-induced endocytosis using confocal microscopy (Fig. 2). BMDCs were incubated with A488-dextran in the presence or absence (control) of 1 µM NA for 3 min, and Rho-ConA was used to stain the cell surface. A488^+
It was reported that NA induces activation of Akt and ERK1/2 in PC12 cells stably expressing each of the three human α₂-AR subtypes but not in wild-type cells (27). However, to the best of our knowledge, no study has reported activation of these molecules in DCs following NA exposure. We next examined the effect of NA treatment on activation of Akt (a downstream effector of PI3K), ERK1/2, and p38 MAPK in DCs. BMDCs were pretreated with 1 μM NA for 3, 7, or 15 min, and the intracellular protein levels of the active forms of these molecules, p-Akt, p-ERK1/2, and phospho-p38 MAPK (pp38), were determined by immunoblotting (Fig. 4A). Low levels of p-Akt, p-ERK1/2, and pp38 were detected in untreated DCs. The level of p-Akt was greatly increased 3 min after NA treatment. The increased level was maintained at 7 and 15 min after NA treatment. The level of p-ERK1/2 also increased 3 min after NA treatment. The p-ERK1/2 level in NA-treated DCs decreased at 7 and 15 min compared with the level at 3 min. In contrast, NA showed little or no effect on the pp38 levels.

We next investigated which receptors were responsible for the activation of Akt and ERK1/2 in DCs. BMDCs were pretreated with prazosin, yohimbine, or propranolol and then treated with NA for 3 min (Fig. 4B, 4C). Prazosin slightly or partially diminished the level of p-Akt or p-ERK1/2 in DCs upon NA treatment. Notably, yohimbine almost completely blocked the NA-mediated increase in the levels of p-Akt and p-ERK1/2. In contrast, propranolol showed no significant effect on the levels of p-Akt and p-ERK1/2. Thus, α₂ ARs seemed to be responsible for Akt and ERK1/2 activation in DCs following NA exposure.

**Effect of PI3K or ERK1/2 inhibition on DC endocytosis upon NA treatment**

To explore the role of PI3K/Akt and ERK1/2 activation following NA exposure, we examined the effect of LY294002, a specific inhibitor of PI3K, and U0126, a specific inhibitor of MEK1/2 (the upstream activator of ERK1/2), on NA-induced endocytosis in DCs. BMDCs were pretreated with LY294002 or U0126 and then treated with 1 μM NA in the presence of each inhibitor for 3 min. The levels of p-Akt and p-ERK1/2 were determined by immunoblotting (Fig. 5A, 5B). LY294002 completely blocked Akt activation upon NA treatment. LY294002 slightly decreased ERK1/2 activation upon NA treatment, although this effect was not statistically significant. In contrast, U0126 completely blocked activation of ERK1/2 upon NA treatment, while showing no effect on Akt activation.

We next analyzed the effect of these inhibitors on DC endocytosis in response to NA. BMDCs were pretreated with LY294002 or U0126 and then incubated with A488-dextran and 1 μM NA for 5 min in the presence of each inhibitor. Flourescence intensity of the cells was analyzed by flow cytometry (Fig. 5C, 5D). LY294002 treatment significantly diminished the NA-induced increase in DC endocytosis, while showing no significant effect on spontaneous DC endocytosis. In contrast, U0126 did not affect spontaneous or NA-induced endocytosis by DCs. Thus, the PI3K pathway, but not the ERK1/2 pathway, seemed to drive NA-mediated DC endocytosis.

**Effect of LPS on NA-induced endocytosis**

It was reported that the endocytic capacity of DCs transiently increased following short-term treatment (30–40 min) with TLR ligands, such as LPS (28). We then examined the influence of LPS treatment on NA-induced endocytosis by DCs. BMDCs were pretreated with LPS for 30 min and then incubated with A488-dextran and NA in the presence of LPS for 3 min (Fig. 6). Similar

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**FIGURE 2.** Morphological evidence for NA-induced endocytosis. BMDCs were incubated with A488-dextran in the presence or absence (control) of 1 μM NA for 3 min. Following fixation and Rho-ConA staining (cell-surface staining), the fluorescence intensity of the cells was analyzed by confocal microscopy. A, Endocytosis in the presence or absence of NA; green, A488-dextran; red, Rho-ConA (original magnification ×40). B, Magnified image of NA-treated cells: A488 image (a), Rho image (b), and merged image (c) (original magnification ×40). Data are representative of three independent experiments.
to previous studies, LPS treatment increased DC uptake of A488-dextran. Of note, NA induced a further increase in endocytosis by DCs upon LPS stimulation. In the presence of yohimbine, NA failed to exert a significant effect on DC endocytosis upon LPS stimulation. Yohimbine alone showed no effect on DC endocytosis upon LPS stimulation. Thus, NA could increase Ag uptake of DCs upon LPS stimulation via a2 ARs.

**Effect of NA on cytokine production by DCs**

It was shown that NA altered the balance of cytokine production in DCs (10–12). Although β AR-mediated regulation is well established, the role of α2 AR-mediated signaling in cytokine production is less consistent. Thus, we examined the influence of NA and the involvement of ARs in IL-12 and IL-10 production by DCs upon TLR stimulation. BMDCs were treated with 1 μM NA and TLR ligands (LPS plus P3C) for 24 h, and cytokine levels in culture supernatant were determined by ELISA. TLR ligands induced IL-12 and IL-10 production by DCs (Fig. 7A). NA completely inhibited IL-12 production by DCs upon TLR stimulation. On the contrary, NA increased IL-10 production by DCs. Prazosin and yohimbine showed no effect on NA-mediated alteration of IL-12 and IL-10 production upon TLR stimulation (Fig. 7B). In contrast, propranolol restored the decreased level of IL-12 production in NA-treated DCs, whereas it inhibited the NA-mediated increase in IL-10 production. Thus, the NA-induced alteration of cytokine production by DCs upon TLR stimulation seemed to be mediated via β ARs but not α1 and α2 ARs.

The β ARs are coupled with G proteins responsible for intracellular cAMP elevation and subsequent PKA activation (16). We next examined the involvement of PKA in NA-mediated alteration of cytokine production by DCs using a PKA-specific inhibitor H89 (Fig. 7C). H89 treatment improved the decrease in IL-12 production caused by NA-treated DCs, whereas it inhibited the NA-mediated increase in IL-10 production. Thus, β AR-mediated...
Discussion

The ARs are G protein-coupled receptors (GPCRs) that are composed of α1, α2, and β ARs that are coupled with Gq, Gi, and Gs proteins, respectively (14–16). Although the effect of NA on cytokine production by DCs has been established (10–12), the influence of this catecholamine on other DC functions is not well documented. It was reported that NA decreases IL-12 production and increases IL-10 production by DCs. NA-induced alteration of cytokine production seems to be mainly mediated via β ARs. The β AR pathway elevates intracellular cAMP level via Gs protein and, thereby, activates PKA (16). PKA activation seems to induce inhibition of IL-12 production and enhancement of IL-10 production in DCs (13). As noted in previous studies, NA modified the balance of IL-12 and IL-10 production by DCs upon TLR stimulation, and β-AR antagonist or PKA inhibition blocked this effect (Fig. 7). It seems that the signal cascade via β ARs, Gs protein, cAMP, and PKA is responsible for the NA-mediated alteration of cytokine production in DCs. In contrast, the role of α2 AR signaling in the immune system is less characterized. In addition to the classical roles of α2 ARs, recent studies showed that α2 AR signaling alters dendritic spine generation in neurons (29, 30). This effect might be responsible for synaptic modification during learning and memory in the central nervous system. The α2 ARs seem to be involved in reconstitution of the cytoskeleton in neurons. In the current study, we found that α2 AR signaling enhanced Ag uptake by DCs following a very short treatment with NA.

The α2 ARs are Gi protein-associated GPCRs and consist of three highly homologous subtypes: α2A-, α2B-, and α2C-ARs. BMDCs were shown to highly express α2A-AR but not α2B- or α2C-ARs (26), suggesting that NA-induced endocytosis is mainly mediated via α2A AR in BMDCs. In the current study, α2 AR-mediated signaling induced PI3K activation and rapid endocytosis in DCs, and blocking PI3K activation inhibited NA-mediated endocytosis. PI3Ks are divided into class I, class II, and class III, based on their structural similarities (31, 32). Class I PI3Ks control many cellular functions, including growth, proliferation, survival, adhesion, and migration. Class I PI3Ks are further divided into class IA (PI3Kα, PI3Kβ, and PI3Kδ) and class IB (PI3Kγ). Of note, PI3Kγ is mainly expressed by immune cells and activated by Gi protein-associated GPCRs, such as chemokine receptors (31). Taken together, we conclude that α2-AR/Gi-protein signaling and subsequent PI3Kγ activation induces DC endocytosis following NA stimulation.

It was reported that PI3K activation upon TLR stimulation is involved in the regulation of cytokine production by DCs (33). Thus, there was a possibility that α2 AR-mediated PI3K activation was also involved in the NA-mediated regulation of cytokine.
production upon TLR stimulation. However, inhibition of the α2-AR pathway, which completely blocked NA-mediated PI3K activation, showed no effect on the NA-mediated alteration of cytokine production. Therefore, NA-mediated PI3K activation is unlikely to be involved in NA-mediated cytokine regulation. It was shown that TLRs activate class IA PI3Ks, whereas GPCRs drive class IB PI3K (PI3Kγ) (31). Thus, the role of α2 AR-mediated PI3K activation seems to be different from that of TLR-mediated PI3K activation in the regulation of DC functions.

Although NA induced significant activation of ERK1/2 via α2 ARs in DCs, blocking the ERK1/2 pathway had no effect on endocytosis by NA-treated DCs (Fig. 5). Thus, the role of ERK1/2 activation in response to NA remains to be elucidated. We are exploring the role of α2 AR-mediated ERK1/2 activation in DC functions.

It was reported that NA suppressed phagocytosis of macrophages via α and β ARs (34). In contrast, some studies showed that NA increased phagocytosis of macrophages, although its effect was modest (35). Therefore, the effect of NA on endocytic activity in macrophages remains controversial. In the previous studies, NA treatment and phagocytosis assay were performed for ≥30 min. To the best of our knowledge, no previous report has shown regulation of endocytosis by NA in DCs. In the current study, we examined the effects of a short treatment (3–20 min) with NA on DC endocytosis and showed that NA notably induced rapid endocytosis via an α2 AR-mediated mechanism in DCs.

In the nervous system, signal transduction is controlled by spatial and temporal regulation of transmitter release, and signaling is terminated by diffusion, rapid reuptake, and degradation. This brief regulation system could apply for the immune system. NA exerted an effect on DC endocytosis within a very short time (3 min). Thus, upon acute stress, brief NA signaling from the sympathetic nervous system might augment Ag capture by DCs to enhance immune responses. It was suggested that very brief stress enhances some aspect of immune function (2). Our present findings may explain, at least in part, the immune enhancement upon acute stress.

The sympathetic nervous system seems to release NA following injury (18). In parallel, the injured tissue could be exposed to pathogenic microbes. It is physiologically possible that DCs are simultaneously exposed to NA and microbe components following injury. TLRs recognize pathogen-associated molecular patterns in the pathogen-derived molecules and are responsible for induction of innate immune responses (36). It was reported that DC facilitates increased Ag uptake following short exposure (30–40 min) to TLR ligands, such as LPS, but not after long-term stimulation (28). The LPS-induced transient enhancement of Ag capture by DCs seems to augment acquired immunity. In agreement with the previous study, brief treatment with LPS enhanced Ag capture by DCs (Fig. 6). In addition, we found that NA induced further enhancement of Ag capture by the LPS-treated DCs. It seems that LPS and NA cooperatively act on the Ag capture by DCs following injury and the subsequent invasion of pathogens.

The relationship between the endocytosis effect and the cytokine effect is an important point to consider with regard to the physiological role of NA in immune responses. Our present findings suggest that NA may induce endocytosis by DCs in peripheral tissue within a few minutes following a stress. Subsequently, DCs are activated by maturational stimuli, such as TLR ligands, and leave the peripheral tissue to migrate into the T cell area of the draining lymph nodes in several hours (37). The NA-mediated alteration in cytokine production by DCs upon TLR stimulation may affect DC-mediated T cell polarization in the lymph nodes, because the effect of NA on cytokine production was detected several hours after NA treatment with TLR ligands (10–12). The cytokine effect might have occurred in T cells and DCs during the Ag presentation in the lymph nodes. It seems that the endocytosis effect or the cytokine effect might be responsible for the quantity or quality of the immune responses, respectively.

In contrast, brief stress may induce temporal release of NA, resulting in short-term exposure of DCs to NA. In contrast, chronic stress may induce prolonged release of NA, resulting in long-term exposure of DCs to NA. Thus, we hypothesize that brief stress may induce the endocytosis effect only, whereas chronic stress may induce the endocytosis effect and the cytokine effect. This hypothesis seems to be compatible with previous studies showing that brief stress enhances some aspects of immune functions, whereas chronic stress induces immune suppression (4, 5).

In this study, we demonstrated a role for NA in the regulation of Ag capture by DCs. Because the endocytosis of exogenous Ags by DCs is essential for Ag presentation to T cells and the induction of adaptive immunity against the microorganisms, further elucidation of the mechanism underlying the NA-mediated regulation of DC endocytosis will lead to the development of clinical applications exploiting this regulation system for the treatment of various infectious diseases and immune disorders.
Disclosures
The authors have no financial conflicts of interest.

References

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