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Butyrate reduced lipopolysaccharide-mediated macrophage migration by suppression of Src enhancement and focal adhesion kinase activity☆

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Abstract

Macrophage motility is vital in innate immunity. Lipopolysaccharide (LPS)-mediated macrophage migration requires the enhancement of Src expression and enzymatic activity, which can be regulated by inducible nitric oxide synthase (iNOS). As a major short-chain fatty acid with histone deacetylase (HDAC) inhibitor activity, butyrate exerts anti-inflammatory effect by regulating the expression of cytokines. However, the influence of butyrate on macrophage movement was vague. In this study, we observed that butyrate inhibited migration of both RAW264.7 and rat peritoneal macrophages elicited by LPS. Unlike its myeloid relatives (i.e. Lyn, Fgr and Hck) whose expression was almost unaltered in the presence or absence of butyrate in LPS-treated macrophages, LPS-mediated Src induction was greatly suppressed by butyrate and that could be attributable to reduced level of the *src* transcript. Similar phenomenon was also detected in LPS-treated macrophages exposed to another HDAC inhibitor, trichostatin A (TSA). Consistent with the indispensability of iNOS in promoting macrophage mobilization via Src up-regulation and the activation of both Src and FAK, we did observe concomitant decrement of iNOS, Src and the suppressed activity of Src and FAK in butyrate- or TSA-pretreated macrophages following LPS exposure. These results imply that by virtue of reduction of Src, butyrate could effectively hamper LPS-triggered macrophage locomotion.

Keywords: Butyrate; Lipopolysaccharide; Src; FAK; macrophage migration

1. Introduction

Macrophages are essential in innate immunity. Activated macrophages secrete a variety of inflammatory mediators including nitric oxide (NO) and cytokines, which in turn contribute to their effector functions such as recruitment and activation of cells involved in immune response. Because macrophages are able to eliminate opsonized pathogens through diverse surface receptors and through antigen presentation to cells of the adaptive immunity, their recruitment to sites of infection turns to be an important physiological process in host defenses. Disturbed regulation of this event leads to pathological disorders such as sepsis and atherosclerosis.

Cellular Src is a nonreceptor tyrosine kinase, which serves as the archetype for a group of Src family tyrosine kinases (SFKs) members including Fyn, Yes, Fgr, Hck, Lyn, Lck and Blk [1]. Mounting evidence indicates that members of SFKs function as cotransducers of transmembrane mitogenic signaling in cells of hematopoietic lineage [2]. Despite Fgr, Hck and Lyn are the predominant SFKs in macrophages, their absence still retains full LPS responsiveness [3] suggesting the presence of one or more elusive, compensating tyrosine kinase(s). Interestingly, the expression of these myeloid-specific SFKs seems almost unaltered in response to lipopolysaccharide (LPS) [4]. In contrast, the expression of Src exhibits a remarkable LPS-inducible manner and promotes macrophage migration [4,5].

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Focal adhesion kinase (FAK) is a well-documented substrate of Src [6]. Its participation in cell–matrix interaction and integrin signaling results in cell spreading, migration and survival [7]. Upon integrin activation, FAK becomes activated and autophosphorylated at Tyr397 that confers the binding site for Src [6]. This accelerates Src-mediated FAK phosphorylation on several tyrosine residues including Tyr861, whose phosphorylation boosts FAK enzymatic activity [6]. The deterioration of movement in macrophages devoid of FAK corroborated the essential role of FAK in macrophage locomotion [8]. Recently, we demonstrated that Src, but not other myeloid-specific SFKs, was required for LPS-induced FAK activation. In agreement with Src induction, FAK activation was inducible nitric oxide synthase (iNOS)-dependent and critical in LPS-mediated macrophage mobility [4].

As the principal anaerobic bacterial fermentation products of dietary fibers, short-chain fatty acids (SCFAs) act as physiological regulators of homeostasis of colonic epithelial cells [9] and are circulated in blood at concentrations in the 1–2-mM range [10]. Among the three most common SCFAs (butyrate, propionate and acetate), butyrate seems to mediate the most profound protective effect within the colon. In addition to playing a critical role in maintenance of a healthy mucosa [11,12], butyrate also induces differentiation, cell cycle arrest and/or apoptosis in a spectrum of tumor cell lines [13–15]. These findings provide rationales for the reported chemopreventive action of high fiber diets toward intestinal tumors [16]. Intriguingly, butyrate also retained anti-inflammatory effects [17,18] and turned to be effective in treating mucosal inflammation in humans [19] and in animal [20] models of colitis.

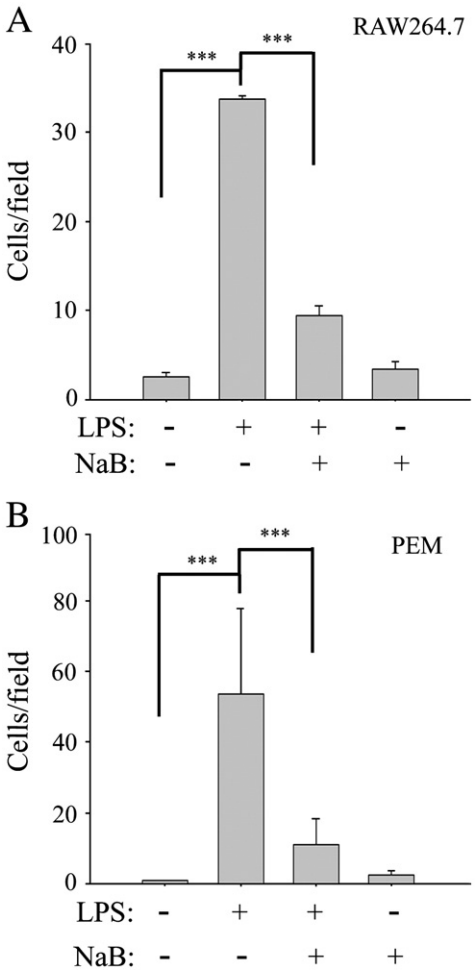

Fig. 1. Butyrate inhibited LPS-mediated macrophage migration. (A) RAW264.7 macrophages and (B) rat PEMs were pretreated without or with sodium butyrate (NaB; 2 mM) for 1 h, and then cells were stimulated without or with LPS for 48 h. The migratory ability of each group was determined by using a Boyden chamber as described under "Methods and materials." Similar results were repeated at least three times and the representative was demonstrated. ***$P<.001$.

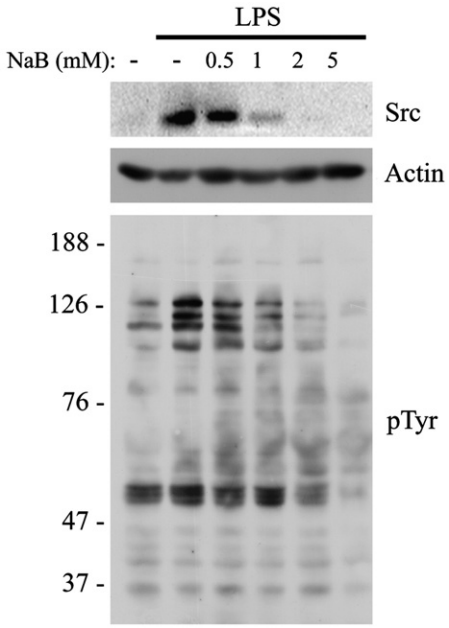

Fig. 2. Butyrate treatment reduced the level of Src and total tyrosyl phosphorylated proteins in LPS-stimulated macrophages. RAW264.7 macrophages were preincubated without or with various concentration of butyrate (0.5, 1, 2, 5 mM) for 1 h, and then cells were stimulated without or with LPS for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. Similar results were repeated three times, and the representative was demonstrated.

Butyrate is a noncompetitive, reversible inhibitor of histone deacetylases (HDACs) [21]. Its modulation of chromatin structure through histone hyperacetylation can alter the expression of a variety of proteins that culminated in changes in cell membrane, cytoskeleton, and cell cycle [22]. The prominent examples include E-cadherin [23], c-Myc [24] and $p21^{CIP1/WAF1}$ [25]. Notably, Src is also a major target of HDAC inhibitors [26]. In this study, we presented evidence demonstrating that butyrate hindered LPS-induced migration in both RAW264.7 and rat peritoneal macrophages (PEMs). And this could be attributable to hampered Src induction and greatly reduced activity of Src and FAK by butyrate. Considering that macrophage migration is important in inflammation, the abrogation of LPS-mediated Src up-regulation by butyrate might have therapeutic potential for diverse inflammatory diseases.

2. Methods and materials

2.1. Reagents and antibodies

Sodium butyrate, trichostatin A (TSA) and LPS purified from *Escherichia coli* serotype 0111:B4 were obtained from Sigma (St. Louis, MO, USA). Thioglycollate was obtained from Merck (Darmstadt, Germany). The primary antibodies used were actin, iNOS, Pi-Tyr397 FAK, FAK (Upstate); Pi-Tyr397 FAK, Pi-Tyr861 FAK, Pi-Tyr416 Src (BIOSOURCE International); Lyn, Fgr, Hck and horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody (PY20) (Santa Cruz). Src-specific mouse monoclonal antibody (2-17) was provided by Dr. Sarah Parsons in University of Virginia.

2.2. Animals

Rats (Sprague–Dawley) were utilized to prepare peritoneal macrophages (PEMs). Environmental conditions as well as light–dark cycles (12:12 h) were controlled. All

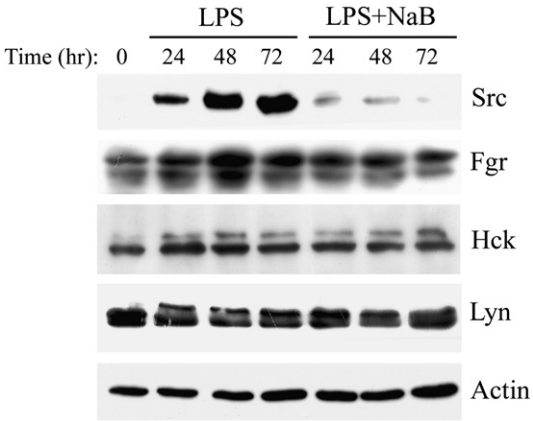

Fig. 3. LPS-elicited Src induction was butyrate sensitive. RAW264.7 cells were preincubated without or with butyrate (2 mM) for 1 h and then cells were stimulated without or with LPS for 24, 48 and 72 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. Similar results were repeated three times and the representative was demonstrated.

experiments using laboratory animals were done in accordance with China Medical University guidelines.

2.3. Cell culture and collection of PEMs

The murine macrophage cell line, RAW264.7 (American Type Culture Collection), was cultured and propagated in RPMI 1640 medium supplemented with 10% fetal calf serum (fetal calf serum [FCS]; HyClone, Logan, UT) and 2 mM L-glutamine at 37°C in humidified atmosphere of 5% CO_2 and air. PEMs were collected by peritoneal lavage from rats (Sprague–Dawley) given an intraperitoneal injection of 8 ml of thioglycollate broth 4 days before harvest. The PEMs were washed with Ca^{+2}- and Mg^{+2}-free phosphate-buffered saline (PBS) and plated in FCS-containing RPMI medium (Rosewell Park Memorial Institute-1640) overnight. Then the cells were washed with medium to remove nonadherent cells, and according to morphological and phagocytic criteria, the resultant macrophage monolayer was >98% pure and ready for experiment.

2.4. Lysate preparation and immunoblot analysis

Lysis of the cells was carried out with modified RIPA buffers as described before [27], and protein concentration was determined by protein assay kit (Bio-Rad) (Hercules, CA, USA). The cell lysates were resolved in an 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel, transferred to nitrocellulose membranes and probed with respective antibodies followed by HRP-conjugated protein A or HRP-conjugated secondary antibodies and detected by Enhanced Chemiluminescence method (Amersham) (Rockford, IL, USA).

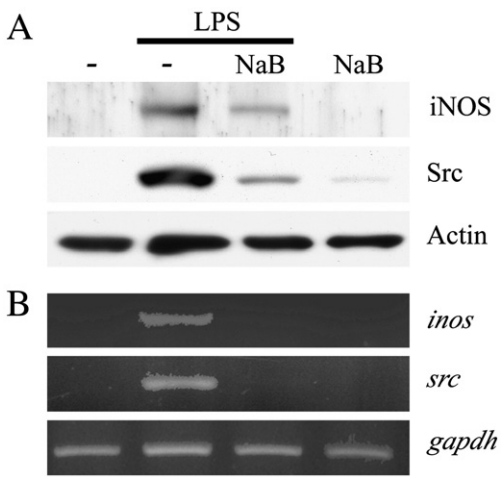

Fig. 4. Butyrate reduced *inos* and *src* transcripts in LPS-stimulated macrophages. RAW264.7 cells preincubated without or with butyrate for 1 h, and then cells were stimulated without or with LPS for 48 h. (A) Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. (B) Meanwhile, the amounts of *inos* and *src* transcripts were analyzed by RT-PCR. *gapdh* was utilized as an internal control for amplification efficiency. For all these experiments, similar results were repeated three times and the representative was demonstrated.

2.5. Reverse transcriptase-polymerase chain reaction

The amount of *src*, *inos* and *gapdh* transcripts was semiquantitated by reverse transcriptase-polymerase chain reaction (RT-PCR) as previously described [15]. The following program is for *src*, *inos* and *gapdh* PCR reaction: the cDNA was denatured for 3 min at 95°C and amplified for 30 cycles under the following conditions: 95°C, 30 s; 60°C, 30 s and 72°C, 45 s, followed by a 5-min elongation step at 72°C. Sequences of primer pairs used were as follows: *src*: forward, 5′-CTGCTGGACTTTCTCAAGGG-3′; reverse, 5′-GTACAGAGCAGCTTCAGGGG-3′; *inos*: forward, 5′-GCCTTCAACAC-CAAGGTTGTCTGCA-3′; reverse: 5′-TCATTGTACTCTGAGGGCTGACACA-3′; *gapdh*: forward, 5′-CCATCACCATCTTCCAGGAG-3′; reverse, 5′-CCTGCTTCACCACCTTCTTG-3′. PCR products were resolved in 2% agarose gel and detected by ethidium bromide staining.

2.6. Transwell migration and time-lapse video microscopy

The migration of cells of interest exposed to LPS was determined by modify Boyden chamber as described before [4]. Briefly, cells were preincubated without or with butyrate (or TSA) for 1 h, and then cells were stimulated without or with LPS for 48 h. Later, cells of each group were added to the upper wells (48-multiwell Boyden microchambers) at 2×10^4 cells per well. The migrated cells will traverse a

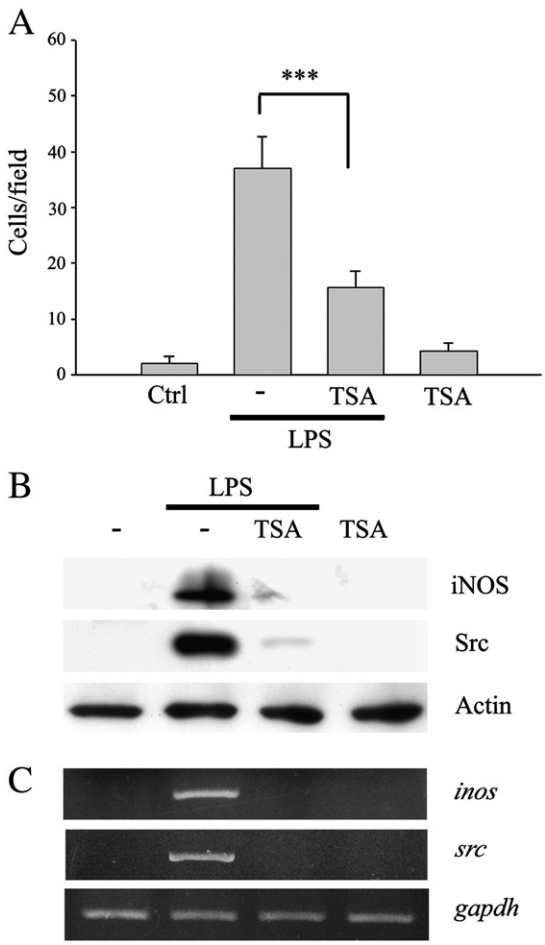

Fig. 5. TSA suppressed migration and reduced *inos* and *src* transcripts in LPS-stimulated macrophages. RAW264.7 cells preincubated without or with TSA (50 ng/ml) for 1 h and then cells were stimulated without or with LPS for 48 h. (A) The migratory ability of each group was determined by using a Boyden chamber as described under Methods and materials. ***$P<.001$. (B) Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. (C) As described above, the amounts of *inos* and *src* transcripts were analyzed by RT-PCR, and the level of *gapdh* was utilized as an internal control for amplification efficiency. The immunoblotting and PCR experiments were repeated three times with similar results, and the representative was demonstrated.

polycarbonate filter (8 μm) from the upper chamber to the lower chamber, which contains 10% FBS as a chemoattractant. After 5 h at 37°C in 5% CO_2, nonmigratory cells on the upper membrane surface were removed with a cotton swab and the cells that traversed and spread on the lower membrane surface were fixed with methanol and stained with Giemsa stain (modified solution) (Sigma). By utilizing a microscope with a 40× objective, the number of migratory cells per membrane was enumerated. Four random fields in each filter were examined. Each experiment was performed in triplicate and migration was expressed as the mean±S.D. of total cells counted per field. Also, the motility of RAW264.7 cells could be monitored using Leica AS MDW system equipped with a Coolsnap HQ camera (Roper Scientific) [4]. Video images were collected at intervals of 20 min for 3 h. The positions of nuclei were tracked to quantify cell motility and analyzed with Metamorph Software (Universal Imaging).

2.7. Statistical analysis

Each experiment was performed three times. The results were presented as means ±S.D. The significance of difference was assessed by Student's *t* test. Bonferroni correction was used for controlling type I error in multiple comparisons.

3. Results

3.1. Butyrate inhibits lipopolysaccharide-induced macrophage migration

LPS is well documented to elicit a variety of cellular activities including cell mobilization in macrophages [4,28]. To examine the effect of butyrate on macrophage motility, the migratory potential of RAW264.7 macrophages treated without or with butyrate (2 mM) prior to LPS exposure was determined. As shown in Fig. 1A, compared to control, significantly increased migration was detected in LPS-stimulated RAW264.7, and this LPS-exerted response could be hampered by butyrate. To further confirm that this butyrate-deteriorated effect could also be applied to primary macrophages, thioglycolate-elicited rat PEMs were utilized to address this issue. As exhibited in Fig. 1B, butyrate significantly suppressed LPS-elicited mobility in rat PEMs.

3.2. Butyrate suppresses Src induction in LPS-stimulated macrophages

Since tyrosyl phosphorylation plays a pivotal role in cell migration, we investigated the influence of various concentrations of butyrate on the profile of tyrosyl-phosphorylated proteins in RAW264.7 after 48 h of LPS treatment. Whole cell extracts prepared from LPS-exposed RAW264.7 without or with butyrate pretreatment were resolved in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by pTyr Western immunoblotting. As depicted in Fig. 2, butyrate caused the decrease in the pTyr content of a number of cellular proteins in a dose-dependent manner, suggesting that butyrate might inhibit one or multiple tyrosine kinases involved in macrophage movement. Given that Src has been demonstrated to be induced, activated and involved in LPS-mediated macrophage mobilization [4], the effect of butyrate on its expression in LPS-exposed macrophages was attempted. As illustrated in Fig. 2, LPS induced the expression of Src and butyrate reduced this enhancement. As a previous observation [5], LPS increased Src expression in a time-dependent manner (Fig. 3). However, butyrate abrogated Src induction by LPS within 24 h (Fig. 3). Notably, the expression of the myeloid-specific SFKs (i.e., Fgr, Hck and Lyn) was almost unaltered and turned to be butyrate-insensitive (Fig. 3).

3.3. LPS-augmented Src transcript is suppressed by butyrate and trichostatin A

Because butyrate could down-regulate the expression of Src, we thereby wondered whether butyrate could affect the abundance of

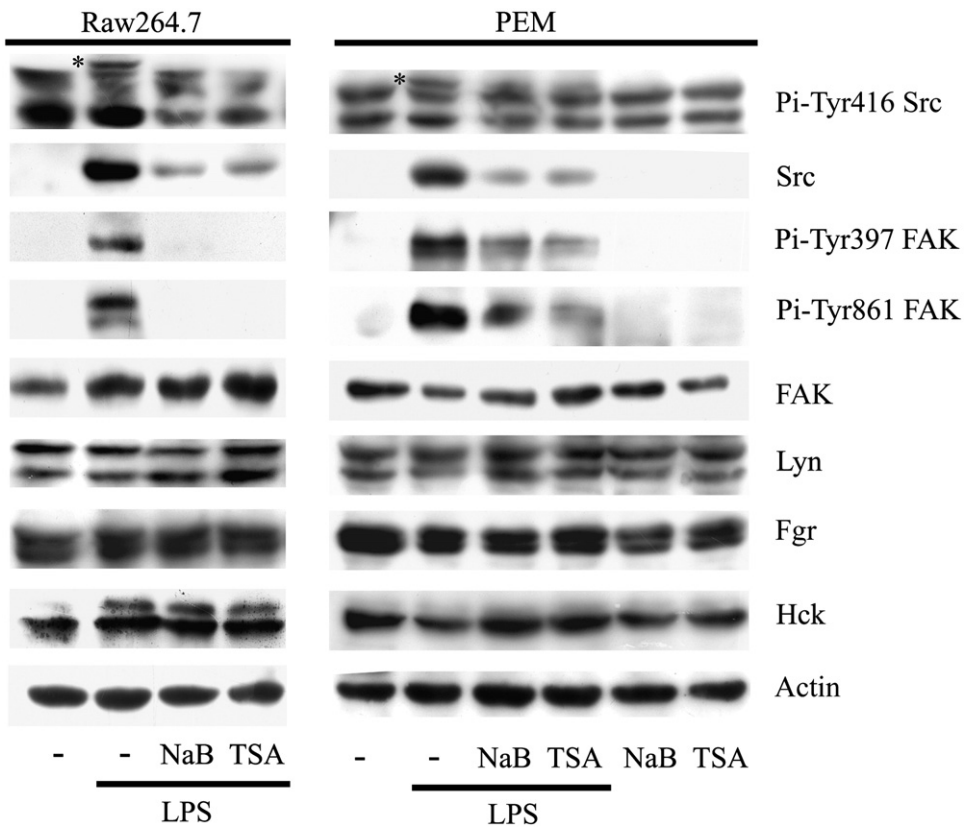

Fig. 6. Butyrate and TSA inhibited LPS-mediated FAK activation in macrophages. RAW264.7 macrophages (left) and rat PEMs (right) were preincubated without or with HDAC inhibitors (i.e., butyrate or TSA) for 1 h, and then cells were stimulated without or with LPS for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The position of Pi-Tyr416 Src is marked with an asterisk. For all these experiments, similar results were repeated three times and the representative was demonstrated.

src transcript. To address this point, total RNAs extracted from control and LPS-exposed RAW264.7 without or with butyrate pretreatment were analyzed by reverse transcription-polymerase chain reaction (PCR). As demonstrated in Fig. 4B, consistent with reduced expression of iNOS and Src in LPS-stimulated RAW264.7 (Fig. 4A), butyrate treatment led to a decrease in the expected 920-bp *inos* and 273-bp *src* transcripts. Due to butyrate is a HDAC inhibitor, we therefore wondered whether other HDAC inhibitors also exerted the same effect on Src expression in macrophages. To address this issue, TSA, another noncompetitive inhibitor of HDAC [29,30], was utilized to study its effect on motility in LPS-stimulated macrophages. Similar to butyrate, TSA significantly inhibited LPS-evoked migration in RAW264.7 cells (Fig. 5A). Concurrent with its inhibitory effect on cell migration, TSA suppressed the expression of iNOS and Src (Fig. 5B), which was attributable to reduced abundance of *inos* and *src* transcripts in LPS-exposed macrophages (Fig. 5C).

3.4. Butyrate and TSA attenuate LPS-mediated FAK activation

Mounting evidence has revealed that FAK is a substrate of Src and its activation plays an important role in macrophage mobilization [4,8,31]; therefore, the impact of butyrate on LPS-evoked FAK activation was examined. Because Tyr397 was the autophosphorylation site of FAK, and Tyr861 was one of the Src-mediated sites on FAK whose phosphorylation promoted FAK activation, the level of FAK Pi-Tyr397 and Pi-Tyr861 was utilized as an indicator for FAK activity assessment. As shown in Fig. 6 (left), simultaneous

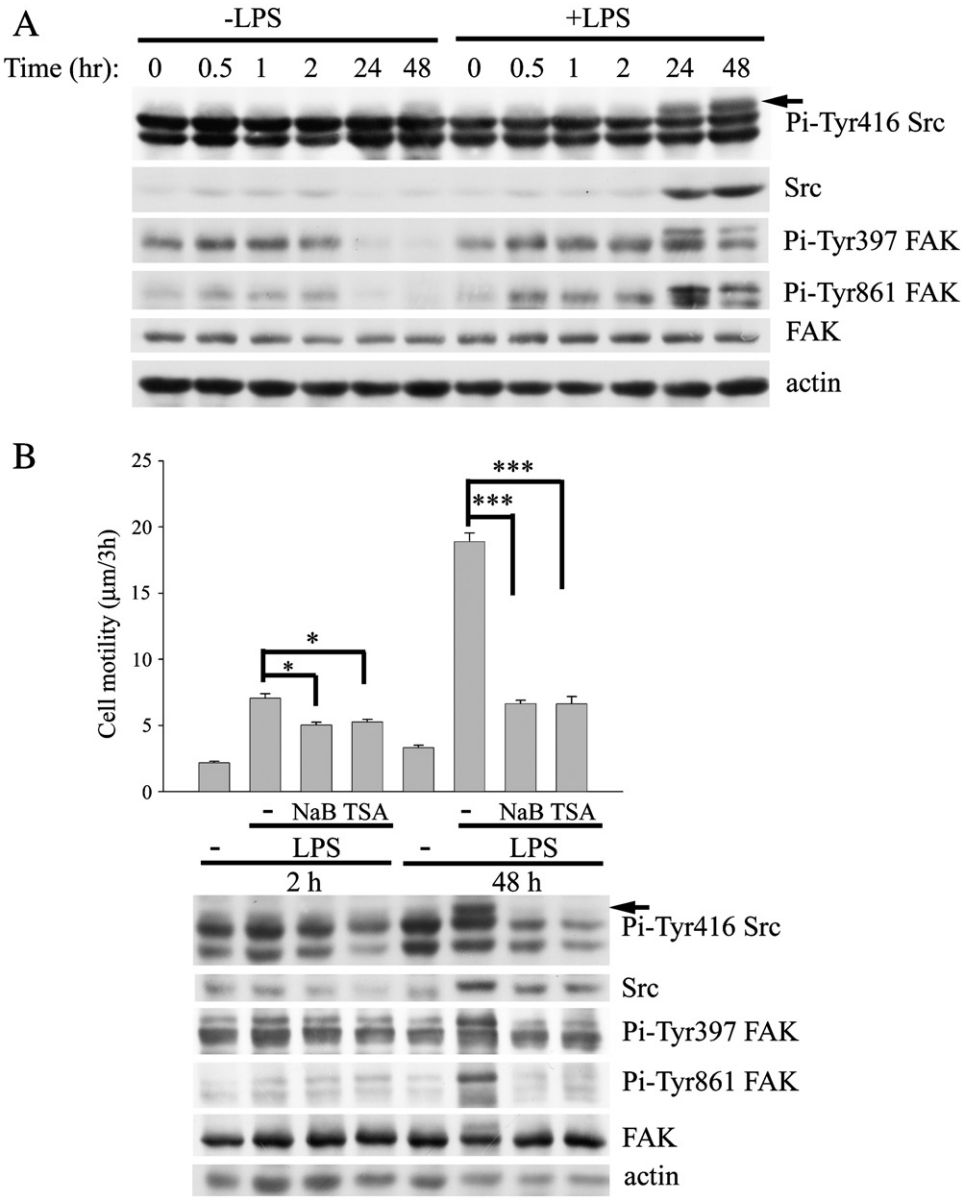

Fig. 7. Effects of butyrate and TSA on migration and Src expression in 2 h LPS-treated RAW264.7 macrophages. (A) RAW264.7 cells were stimulated without or with LPS for various time points (i.e., 0.5, 1, 2, 24 and 48 h). Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. (B) RAW264.7 cells pretreated without or with butyrate or TSA for 1 h, and then cells were stimulated without or with LPS for 2 or 48 h. The motility of each group was measured by time lapse video microscopy as described under Methods and materials and calculated as velocity (μm/3 h) of cells from three fields (>20 cells/field). *P<.05; ***P<.001. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated. The position of Pi-Tyr416 Src is marked with an arrow. For all these experiments, similar results were repeated three times and the representative was demonstrated.

enhancement of Src expression, activation (as reflected by Pi-Tyr416 Src) and elevated FAK activity were detected in LPS-treated RAW264.7 macrophages. However, both butyrate and TSA profoundly inhibited these LPS-mediated events. Unlike the inducible characteristic of Src, the level of myeloid SFKs was unaltered by LPS, and their expression was also unaffected by butyrate and TSA. Importantly, similar phenomenon was also observed in LPS-stimulated rat PEMs that were pretreated with butyrate or TSA (Fig. 6, right). Since cells were stimulated with LPS for 48 h in all the experiments described above, one interesting question prompted was if there was any effect by butyrate treatment in the short-time course in terms of Src/FAK activation. To address this issue, the level of Src, Pi-Tyr416 Src, FAK, Pi-Tyr397 FAK and Pi-Tyr861 FAK in short-term (i.e., 0.5, 1, 2 h) and long-term (i.e., 24 and 48 h) LPS-treated RAW264.7 was determined. Much more, elevated Src, Pi-Tyr416 Src, Pi-Tyr397 FAK and Pi-Tyr861 FAK were detected in long-term LPS-exposed RAW264.7 as compared to that in cells treated with LPS for only short period of time (Fig. 7A). Next, we assessed the effect of butyrate and TSA on the migratory ability and the level of Src, Pi-Tyr416 Src, FAK, Pi-Tyr397 FAK and Pi-Tyr861 FAK in 2- and 48-h LPS-exposed RAW264.7. As exhibited in Fig. 7B, 2 h LPS exposure did result in moderate mobility increase, and the moving distance of 48 h LPS-treated cells was around two- to threefold of that detected in cells with 2 h LPS treatment. Butyrate and TSA significantly impaired cell motility in both cases. Concurrently, both agents decreased Src, Pi-Tyr416 Src, Pi-Tyr397 FAK, and Pi-Tyr861 FAK in 48-h LPS-treated cells while slightly altered Src and FAK activity was detected in cells exposed to LPS for 2 h. With this finding, we concluded that the migratory potential of LPS-stimulated macrophages was greatly relied on Src induction.

4. Discussion

As the target of Src, FAK was required in macrophage motility [8]. Its augmented Pi-Tyr397 and Pi-Tyr861 level caused by Src induction was also LPS-mediated and iNOS-dependent [4]. In this study, we presented evidence indicating that butyrate and TSA hampered LPS-triggered migration in macrophages (Figs. 1, 5A, and 7B). By suppressing LPS-mediated Src induction and activation of both Src and FAK (Figs. 6 and 7B), butyrate and TSA effectively reduced cell motility in RAW264.7 and rat PEMs initiated by LPS (Figs. 1, 5A, 7 and data not shown). The LPS-induced Src expression, FAK activation and cell migration were not obvious in 2 h-treated RAW264.7 as compared to that in cells exposed to LPS for 48 h (Fig. 7). Of note, butyrate exerted its inhibitory action on LPS-evoked Src expression in a dose-dependent fashion (Fig. 2); the amount of Lyn, Fgr and Hck was almost unaltered (Fig. 3). This finding manifested the constitutive expression of the myeloid-specific SFKs and further strengthened the notion that they might play a housekeeping role in macrophage physiology. By contrast, due to its versatile expression profile in response to diverse stimuli, Src was expected to execute certain tasks in macrophages when insults and crises were met.

Concomitant reduction of *src* transcript was observed in LPS-stimulated macrophages preincubated with butyrate (Fig. 4B) and TSA (Fig. 5C). Thus, this butyrate- and TSA-mediated decrease in Src expression could be partly attributable to the suppressed level of its corresponding *src* transcript. Similar parallel decrease of Src mRNA and protein caused by butyrate and TSA was also documented in a spectrum of human cancer cell lines [26]. And surprisingly, butyrate and TSA repressed the activity of two distinct *src* promoters without interfering the binding of protein factors essential for promoter's activity [26]. There are eighteen identified HDACs belonging to four categories, i.e. class I, II (which is further divided into IIa and IIb), III, and IV in humans [32]. Butyrate inhibited the activity of Class I HDACs (1, 2, 3 and 8) and Class IIa HDACs (4, 5, 7 and 9) while TSA decreased the activity of Class I HDACs and Class II HDACs (4, 5, 6, 7, 9, 10) [33]. Given that Class I HDACs are present in the nucleus and Class IIa HDACs can shuttle between nucleus and cytoplasm, the suppression of transcription activity of *src* promoters by butyrate and TSA invites a speculation that a nuclear HDAC (s) is likely the target(s) in this context. Since HDAC inhibitors could also acetylate non-histone targets as well, it was then plausible that down-regulation of the *src* promoters by butyrate and TSA might be achieved by events other than histone acetylation and deacetylation.

As a short chain fatty acid, butyrate has been demonstrated to reduce NO production in LPS-stimulated RAW264.7 macrophages [34]. Here, the reduction of LPS-elicited iNOS not only observed in butyrate-treated macrophages (Fig. 4A) but also detected in cells incubated with TSA, a HDAC inhibitor with hydroxamate moiety (Fig. 5B). Thus, the suppression of LPS-evoked iNOS in macrophages was not limited to a specific class of HDAC inhibitors. Consistent with the indispensability of iNOS, a well-established nuclear factor κB (NF-κB) target, in promoting macrophage mobilization via Src up-regulation and the activation of both Src and FAK, we did observe concomitant decrement of iNOS, Src (Figs. 4A and 5B) and the suppressed activity of Src and FAK (Fig. 6) in butyrate- and TSA-pretreated macrophages following LPS exposure. Based on these findings, we proposed a simple model to illustrate how butyrate- and TSA-mediated inhibition of LPS-triggered macrophage mobilization could be achieved (Fig. 8).

It is now well established that acetylation of histone and nonhistone proteins can directly and indirectly control the duration, strength and specificity of the NF-κB-activating signaling pathway at multiple levels. And in combination with other post-translational protein modifications, acetylation and deacetylation events can generate an "NF-κB-signaling code," which could, by analogy with the "histone code," modulate NF-κB-dependent gene transcription in an inducer- and promoter-dependent manner [35]. Butyrate has been demonstrated to abolish NF-κB activation by blocking the degradation of IκBα and IκBβ [34]. However, considering the complexity of the "NF-κB-signaling code," we wonder if there should be other unidentified, butyrate-elicited events impairing NF-κB activation,

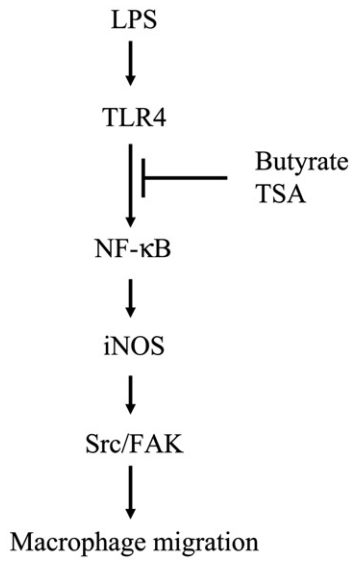

Fig. 8. Model of suppression of LPS-evoked macrophage migration by butyrate and TSA. Combined the published reports and our current studies, we propose that, by impairing NF-κB activation and iNOS induction, butyrate and TSA can suppress the enhancement of Src and activation of FAK that contribute to macrophage mobilization in response to LPS.

iNOS expression, Src enhancement and macrophage locomotion that merit further investigation.

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