International Endodontic Journal

doi:10.1111/j.1365-2591.2010.01773.x

The effect of lysophosphatidic acid and Rho-associated kinase patterning on adhesion of dental pulp cells

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Abstract

Cheng R, Shao M-Y, Yang H, Cheng L, Wang F-M, Zhou X-D, Hu T. The effect of lysophosphatidic acid and Rho-associated kinase patterning on adhesion of dental pulp cells. *International Endodontic Journal*, **44**, 2–8, 2011.

Aim To investigate the effects of lysophosphatidic acid (LPA) and the Rho/Rho-associated kinase (ROCK) pathway on adhesion of dental pulp cells (DPCs).

Methodology Human DPCs were cultured *ex vivo*. After treatment of LPA and Y-27632, a specific ROCK inhibitor, changes in focal contacts (FCs) were examined by immunofluorescent staining. Activation of FCs proteins was examined by measuring tyrosine 397 phosphorylation of focal adhesion kinase (FAK) and paxillin using immunoblotting. The data were analysed by Student's *t*-test.

Results The immunofluorescent staining indicated LPA stimulation induced larger focal adhesion in the cell periphery, compared with the control. Inhibition of ROCK by Y-27632 decreased the formation of FCs markedly, even in the LPA-stimulated cells. LPA also increased the level of tyrosine phosphorylation of paxillin at 30 min ($P < 0.05$) and FAK at 5 and 30 min ($P < 0.05$). Furthermore, p-paxillin levels declined immediately after Y-27632 treatment and remained low at 5, 30, 60 min. Y-27632 also suppressed the effects of LPA on p-paxillin and p-FAK at 5 and 30 min ($P < 0.05$).

Conclusion LPA activated Rho and then subsequently activated ROCK, suggesting that LPA influences the FCs of DPCs by modulating tyrosine phosphorylation of FAK and paxillin via the Rho/ROCK pathway.

Keywords: focal adhesion kinase, lysophosphatidic acid, paxillin, Rho, Rho-associated kinase.

Received 7 January 2010; accepted 18 May 2010

Introduction

When the dental pulp is injured, dental pulp cells (DPCs) react to repair the injury and to resist further damage by maintaining the integrity of pulp and dentine (Goldberg & Smith 2004). During the pulp-repair process, lysophosphatidic acid (LPA), generated by degranulating platelets during blood clot formation, is released at the sites of injured tissue where it might play a role during the subsequent healing process (Sano *et al.* 2002). This bioactive lipid can induce cell proliferation, survival adhesion, migration and gene expression (Moolenaar 1995). Previous studies have shown that LPA is likely to have a mitogenic effect on DPCs, but LPA maintains odontoblast-like cells in an undifferentiated state (Gruber *et al.* 2004). Although it is known that LPA influences the proliferation and differentiation of DPCs, the ability of LPA to induce cell movement and adhesion has not been examined.

Cell movement requires coordinated establishment and detachment of adhesion sites. Cells attach to the extracellular matrix (ECM) by focal contacts (FCs), with different stages occurring during adhesion. At first,

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activated integrin localizes at the tip of the leading edge and interacts with ECM to form nascent adhesions. Later, these nascent adhesions become focal complexes (FXs), which are located mainly at the base of the lamellipodium. The intracellular F-actin is thought to be connected to FXs and to facilitate lamellipodium protrusion. Finally, some FXs mature into the largest form of FC, the focal adhesion (FA). FAs associate with the termini of F-actin and provide cells with contractile forces (Geiger *et al.* 2001, Kaverina *et al.* 2002, Pollard & Borisy 2003, Zaidel-Bar *et al.* 2004, Galbraith *et al.* 2007, Choi *et al.* 2008).

FCs comprise many FA-associated proteins. At the adhesion sites, paxillin and talin connect directly to integrin (Geiger *et al.* 2001). Later, some other proteins such as vinculin, focal adhesion kinase (FAK) and zyxin are recruited to form FXs and FA (Zaidel-Bar *et al.* 2003). In various FA-associated proteins, paxillin and FAK are two important components that function in transmitting signals downstream of integrins. FAK is recruited to the sites of integrin–ECM attachment, inducing autophosphorylation at tyrosine 397 and phosphorylating both FAK and paxillin, thereby initiating intracellular signalling cascades (Playford & Schaller 2004).

Cell migration is regulated largely by the coordination of signalling pathways via integrins (Yamada & Miyamoto 1995, Giancotti & Ruoslahti 1999, Webb *et al.* 2002). The Rho GTPase pathway is one signalling pathway that influences the formation of stress fibres and FAs (Ridley & Hall 1992). Rho, a Rho GTPase member, and its downstream effector Rho-associated kinase (ROCK), have been shown to direct the assembly of FAs (Hall 1994, Narumiya *et al.* 1997). For example, LPA, a Rho activator, induces the Rho-dependent formation of FAs and the phosphorylation of FAK and paxillin in fibroblasts (Ridley & Hall 1992). Rho protein is necessary for the tyrosine phosphorylation of paxillin and FAK in cell types other than fibroblasts (Yano *et al.* 1996, Garcia *et al.* 1997, Etienne *et al.* 1998, Tapia *et al.* 1999).

Previous experiments have indicated that LPA and ROCK play a major role in the migration of DPCs, lamellipodium formation, cytoskeleton assembly and alpha smooth muscle actin expression (Cheng *et al.* 2010). In this study, the effect of LPA on DPCs was investigated by measuring the changes in FCs and tyrosine phosphorylation of FAK and paxillin. To understand the possible signalling pathway better, the specific ROCK inhibitor Y-27632 was used to examine the influence of the Rho/ROCK pathway.

Material and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). Collagenase type I, LPA and Y-27632 (ROCK inhibitor) were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies to FAK, paxillin, FAK phosphorylated at Tyr397 and paxillin phosphorylated at Tyr118 were from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal antibody to vinculin was from Sigma-Aldrich.

Cell culture

Human impacted third molars were collected from three adults (aged 20, 26 and 28) with informed consent according to the institutional standards (Council for International Organizations of Medical Sciences 2002). The pulp tissue was separated and digested in 0.3% collagenase type I for 1 h at 37 °C. Released cells were cultured in DMEM supplemented with 10% FBS. The DPCs were kept in a humidified atmosphere at 37 °C in 5% CO_2. Cells between passages 5 and 8 were used. The cells between passages 5 and 8 were washed twice with phosphate-buffered saline (PBS) and cultured in serum-free DMEM for 24 h. Following this, the cells were treated with 0 or 10 μmol L^{-1} Y-27632 or with 0 or 5 μmol L^{-1} LPA for the indicated times.

Immunofluorescent staining of vinculin

After the treatment mentioned earlier, cells were washed thrice with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were treated with 0.1% Triton X-100 for 5 min and then blocked with PBS with 0.1% Tween 20 (PBST) containing 10% goat serum for 60 min. The blocking solution was removed, and the cells were incubated overnight with anti-vinculin monoclonal antibody at 4 °C. The cells were washed with PBST and incubated with rhodamine-conjugated anti-mouse IgG for 60 min. After extensive washing, the cells were covered by glycerol buffer, and images were obtained using a fluorescence microscope (Carl Zeiss, Göttingen, Germany).

Immunoblotting

Cells were lysed in ice-cold lysis buffer containing 20 mmol L^{-1} Tris–HCl, pH 7.4, 5 mmol L^{-1} EDTA,

150 mmol L^{-1} NaCl, 30 mmol L^{-1} sodium pyrophosphate, 25 mmol L^{-1} NaF, 1% Triton X-100, 100 μmol L^{-1} Na_3VO_4 and 1 mmol L^{-1} phenylmethanesulfonyl fluoride. Lysates were clarified by centrifugation at 12 000 *g* for 10 min at 4 °C. The supernatants were subjected to SDS/PAGE (10% gel), and the proteins were transferred to Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), as described previously (Rehman & Wang 2009). The primary antibodies were anti-FAK, anti-paxillin, anti-FAK phosphorylated at Tyr397, anti-paxillin phosphorylated at Tyr118 (Cell Signaling Technology, Beverly, MA, USA) and anti-GAPDH (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Immunoreactive bands were visualized by an enhanced chemiluminescence kit (Millipore) and quantitated by scanning densitometry using ChemiDoc XRS (Bio-Rad Laboratories Inc., Hercules, CA, USA).

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Statistical analysis

All experiments were performed in triplicates. The data were analysed using Student's *t*-test. The significance level was set at 0.05.

Results

Effect of LPA and ROCK on FCs in DPCs

To understand the effect of LPA and ROCK on DPCs, the distributions of FCs were examined. In control cells, dot-like FCs were distributed mainly in the tip of the cell protrusions (Fig. 1a). Cells treated with LPA showed a different location and shape of FCs. Some larger FAs appeared in the cell periphery, indicating that LPA induced the FA formation (Fig. 1b). Inhibition of ROCK by Y-27632 decreased the formation of FCs markedly (Fig. 1c), even in the LPA-stimulated cells (Fig. 1d). However, some small FXs appeared in the base of the lamellipodium. These results suggest that ROCK inhibition suppressed the maturation of FXs into FAs.

Influence of LPA and ROCK on tyrosine phosphorylation of FA proteins FAK and paxillin

To address the mechanisms by which LPA and ROCK exert their effects on cell FA, their influences on FAK and paxillin were examined. FAK and paxillin are two important proteins that localize to FAs. To determine the time course of LPA-induced phosphorylation, cells were incubated for 5, 30 or 60 min. Tyrosine phosphorylation of FAK and paxillin increased transiently and peaked 5 min after stimulation, decreased somewhat at 30 min after stimulation and then increased again at 60 min (Fig. 2a). Densitometric analysis indicated that p-FAK increased during LPA stimulation at 5 and 30 min ($P < 0.05$) (Fig. 2b). An increase in phosphorylation of paxillin was also observed at 30 min ($P < 0.05$) (Fig. 2c). LPA also stimulated a high level of p-FAK and p-paxillin at other times, although these were not significant increases.

Moreover, it was observed that p-paxillin levels declined immediately after ROCK inhibition and remained at this low level during the entire time course. Y-27632 also decreased the effect of LPA on p-paxillin ($P < 0.05$). As shown in Fig. 2, although Y-27632 did not affect the quantity of p-FAK, it inhibited the LPA-induced tyrosine phosphorylation of FAK at 5 and 30 min ($P < 0.05$). These findings suggest that the Rho/ROCK pathway is essential for the LPA-induced tyrosine phosphorylation.

Discussion

LPA induces Rho-dependent FA formation (Buhl *et al.* 1995), but no study has focused on the effects of this lipid growth factor and on DPCs. The immunofluorescent staining of vinculin, a FA-associated protein widely distributed in fibroblast, revealed the adhesion sites. LPA was found to activate Rho to induce the formation of larger FAs than in the control condition. Previous studies have shown that FCs are assembled by various FA proteins through a hierarchical process. It can be speculated that more proteins were recruited into the larger FAs (Zaidel-Bar *et al.* 2004). By contrast, the inhibition of ROCK by Y-27632 caused DPCs to lose the dot-like FCs and prevented the LPA-induced increase in FA formation. The vinculin immunofluorescence staining indicated that Y-27632 prevented the recruitment of free vinculin to the FCs. As a result, small FXs located in the base of the lamellipodium could not mature into FAs. These results suggest that LPA promotes FC formation, whereas ROCK inhibition suppresses the effect of LPA.

To illuminate the mechanism, how LPA and ROCK altered the assembly of FCs by measuring tyrosine phosphorylation of FAK and paxillin was examined. FAK is a non-receptor tyrosine kinase that is associated with cell adhesion, which involves close contact between the cell and the ECM. FAK is a key mediator of this response, and its tyrosine phosphorylation leads

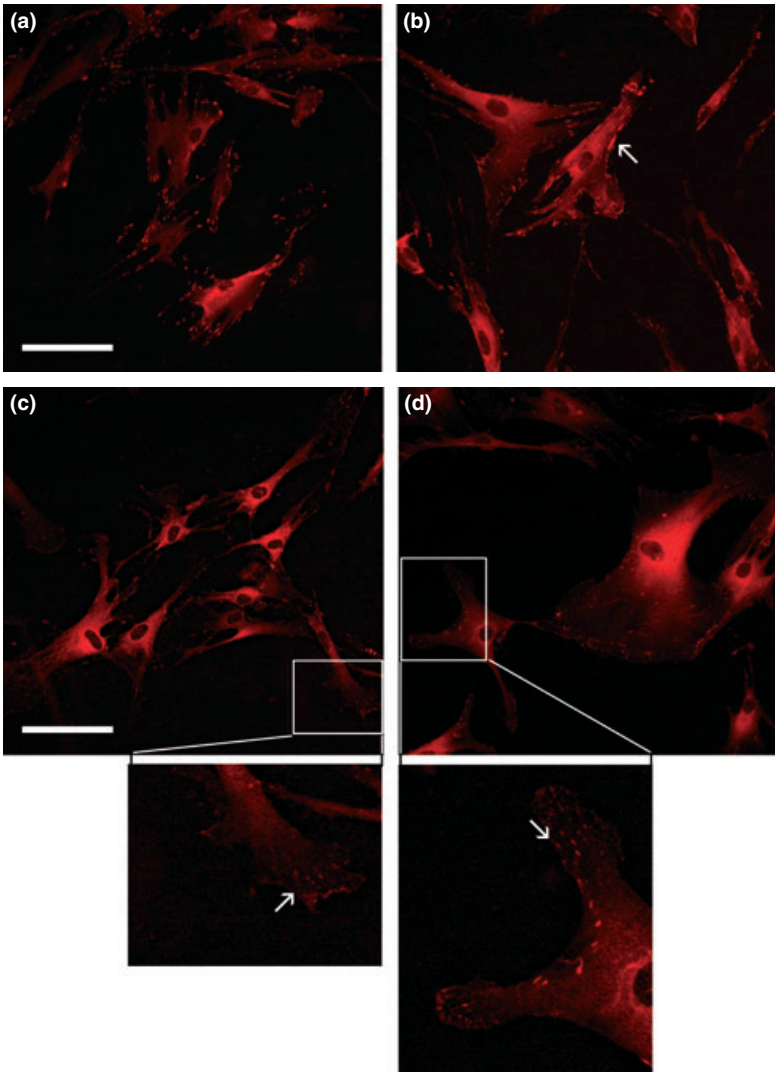

Figure 1 Lysophosphatidic acid (LPA) and Rho-associated kinase (ROCK) influence focal contacts (FCs) in dental pulp cells (DPCs). FCs were stained with anti-vinculin antibody. Scale bar = 100 μm. (a) Dot-like FCs were seen in the control cells. (b) Stimulation with LPA (5 μmol L^{-1}) for 60 min resulted in larger FAs than in the control, especially in the periphery of the cell (arrow). (c) When cells were treated with Y-27632 for 60 min to block the ROCK pathway, most dot-like FCs disappeared, whereas the cytoplasm contained a high level of free protein. (d) Y-27632 inhibited the effect of LPA on DPCs. (c) and (d) show a higher magnification (×9) of a lamellipodium protrusion, indicating the newly recruited vinculin in small FCs (FXs, arrow).

to the recruitment and activation of Src. Src activation phosphorylates some downstream effector such as paxillin, thereby initiating intracellular signalling cascades (Hildebrand *et al.* 1995, Parsons 2003, Mitra & Schlaepfer 2006).

Paxillin is also a binding protein for the FCs and the actin-binding protein vinculin (Turner *et al.* 1990). It functions as a link between the actin filaments and the integrin-rich cell adhesion sites. Integrin is associated with paxillin tyrosine phosphorylation through activation of FAK and Src (Schaller & Parsons 1995, Wade *et al.* 2002).

In many types of cells, tyrosine phosphorylation of FA proteins occurs under various stimuli, which use different signalling pathways. As reported previously (Tsuji *et al.* 2002, Le Boeuf *et al.* 2006), phosphorylation of paxillin or FAK is mediated by the ROCK pathway in fibroblasts and endothelial cells. In addi-

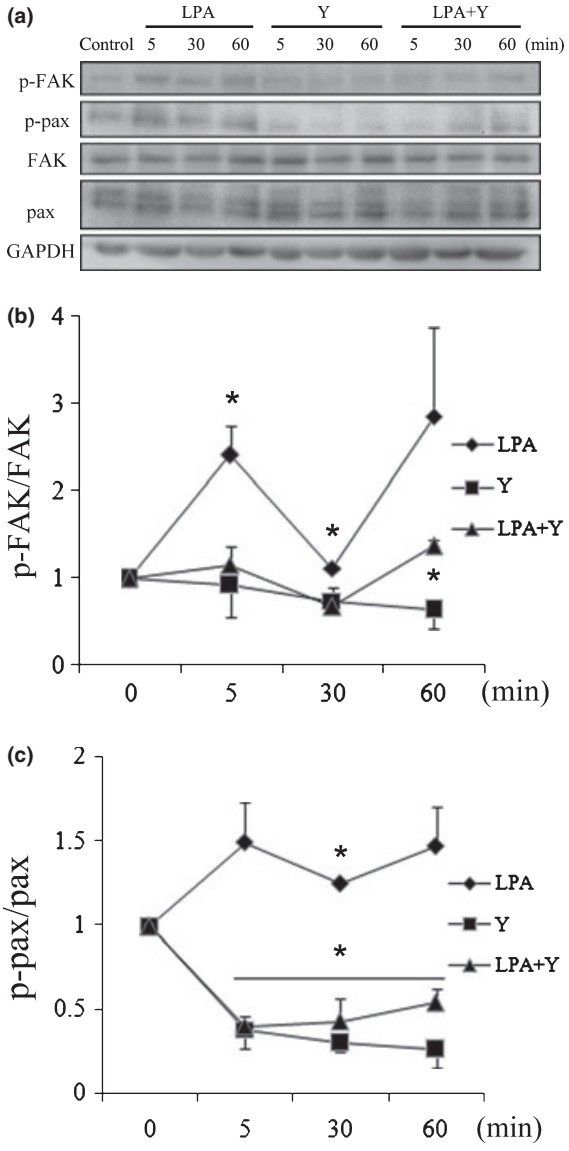

Figure 2 Lysophosphatidic acid (LPA) and Y-27632 influence phosphorylation of focal adhesion kinase (FAK) and paxillin. (a) Serum-starved cells were stimulated with LPA or Y27632 or both for 5, 30 or 60 min. Phosphorylated and total FAK and paxillin phosphorylation were assessed using antibodies as described in Materials and methods. (b) and (c) Densitometric analysis of four Western blot experiments performed with antibodies against p-FAK and p-paxillin normalized by total FAK and paxillin, respectively. The data were obtained from three experiments, $^*P < 0.05$. p, phosphorylated; Y, Y-27632.

tion, LPA induces Rho/ROCK-dependent movement of FAs and the trailing-edge of fibroblasts (Iwanicki *et al.* 2008).

Similar to results shown in other cell types, the present data revealed that LPA induced the activation of FAK and paxillin in DPCs. Inhibition of ROCK with Y-27632 suppressed the tyrosine phosphorylation of paxillin. Y-27632 also stimulated a decline in p-FAK and p-paxillin during exposure of the cells to LPA, suggesting that the Rho/ROCK pathway participates in the tyrosine phosphorylation of FAs. These results suggest that the signalling occurs between the Rho/ROCK pathway and FA proteins and this induces the tyrosine phosphorylation of paxillin and FAK.

Y-27632 disturbed the ability of paxillin to connect FCs to vinculin, thus inhibiting the formation of FAs. Inhibition of ROCK did not suppress tyrosine phosphorylation of FAK compared with the control cells which had a low level of p-FAK. However, in untreated cells the p-paxillin level was correspondingly higher than that of p-FAK, which maintained normal cell adhesion function. Thus, some other mechanism might be involved.

Some growth factors also appear to function via a range of transmembrane receptor families to directly or indirectly trigger paxillin tyrosine phosphorylation (Rankin *et al.* 1996, Riedy *et al.* 1999, Tapia *et al.* 1999, Schaller 2001). These growth factors provoke significant changes in the organization of the cytoskeleton or the state of cellular proliferation. LPA reacts as a bioactive lipid and mediates many cell functions including proliferation, adhesion, migration, differentiation and survival (Goetzl 2001). The study implicated that LPA mediated adhesion of DPCs and contributes to dental pulp healing. Further investigations into the effects of LPA and its direct or indirect signalling pathways in triggering pulp repairing activity might provide some new methods to preserve injured dental pulp.

Conclusion

In summary, these data suggest that LPA modulates FCs in DPCs by inducing tyrosine phosphorylation of FAK and paxillin via the Rho/ROCK pathway.

Acknowledgements

The authors thank M Zhou for assistance with fluorescence microscopy. This work was supported by the National Natural Science Foundation of China (30973322) and the Research Fund for the Doctoral Program of Higher Education of China (20090181110089).

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