LY294002 inhibits monocyte chemoattractant protein-1 expression through a phosphatidylinositol 3-kinase-independent mechanism

Eun-Kyoung Choi[a], Hyun-Ju Park[b], Jae-Sook Ma[b], Hyun-Chul Lee[c], Ho-Cheol Kang[d], Byung-Gook Kim[a], In-Chol Kang[a,*]

[a] *Dental Science Research Institute, Chonnam National University, Kwangju 500-757, South Korea*
[b] *Department of Pediatrics, Chonnam National University Medical School, Kwangju 501-190, South Korea*
[c] *Department of Microbiology, Chonnam National University Medical School, Kwangju 501-190, South Korea*
[d] *Department of Internal Medicine, Chonnam National University Medical School, Kwangju 501-190, South Korea*

Received 2 November 2003; revised 8 January 2004; accepted 8 January 2004

First published online 23 January 2004

Edited by Beat Imhof

Abstract The effects of LY294002 (LY29) and wortmannin (WM), inhibitors of phosphatidylinositol 3-kinase (PI3K), on monocyte chemoattractant protein-1 (MCP-1) expression by human umbilical vein endothelial cells were investigated. Complete inhibition of interleukin (IL)-1β-induced Akt phosphorylation occurred at 50 μM LY29 or 100 nM WM. At these concentrations, LY29, but not WM, significantly inhibited constitutive and IL-1β-induced MCP-1 expression at both protein and mRNA levels. LY303511 (LY30), an inactive analogue of LY29, also inhibited MCP-1 expression. LY29 and LY30 inhibited activation of nuclear factor-κB (NF-κB). These results suggest that LY29 inhibits MCP-1 expression at least in part via suppression of NF-κB, independent of PI3K, and the structure of LY29 and LY30 may be a novel template for development of new anti-inflammatory drugs.
© 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Monocyte chemoattractant protein-1; Phosphatidylinositol 3-kinase; LY294002; LY303511; Nuclear factor-κB

1. Introduction

LY294002 (LY29) and wortmannin (WM), specific inhibitors of phosphatidylinositol 3-kinase (PI3K), have been widely used to examine the role of PI3K in cellular responses. LY29 and WM are structurally distinct compounds having working concentrations of around 50 μM and 100 nM, respectively, for cell-based assays [1–4]. Although the working concentration of LY29 is about 500-fold higher than that of WM, LY29 is widely used in cell biology as a specific PI3K inhibitor because it is much more stable in solution than WM [5].

In any studies with inhibitors, there is a possibility that targets other than the desired one may be affected. Indeed, LY29 and WM can act via additional targets besides PI3K. For example, while LY29 inhibited lipopolysaccharide-stimulated nitric oxide production in Raw 264.7 macrophage cells, WM was ineffective [6]. In contrast, only WM significantly inhibited activation of mitogen-activated protein kinase in interleukin (IL)-3-stimulated MC-9 mast cells [7]. However, there has been no report on the differential effects of the two inhibitors on cytokine production.

During our research into the intracellular signaling pathways of *Porphyromonas gingivalis*-induced monocyte chemoattractant protein-1 (MCP-1) production in endothelial cells, we found that LY29, but not WM, potently inhibited *P. gingivalis*-induced MCP-1 mRNA expression, which led us to conduct further experiments on the MCP-1-inhibitory effect of LY29 using IL-1β as an MCP-1 inducer. MCP-1, a CC chemokine, plays important physiologic and pathogenic roles by recruiting monocytes. In innate immunity, monocytes are recruited from the blood to sites of infection by MCP-1. A major role for MCP-1 in atherogenesis is well established. In response to atherogenic stimulants including IL-1β, MCP-1 is induced in endothelial cells and promotes the development of atherosclerosis [8].

In this study, we report for the first time that the widely used PI3K inhibitor, LY29, and its inactive analogue LY303511 (LY30) inhibit IL-1β-stimulated MCP-1 production by endothelial cells without involving PI3K.

2. Materials and methods

2.1. Chemicals and cell culture

LY29, LY30, and WM were purchased from Sigma (St. Louis, MO, USA). LY29 and WM were dissolved in a 1:1 mixture of dimethylsulfoxide and ethanol. LY30 was dissolved in water. Human recombinant IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). Unless stated otherwise, all chemicals were from Sigma. Primary human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Walkersville, MD, USA) and used at passages 3–6. The cells were cultured in Ham's F-12K medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Life Technologies), 25 μg/ml endothelial cell growth supplement, 100 μg/ml heparin, and 50 μg/ml gentamicin at 37°C in 5% CO_2.

2.2. MCP-1 enzyme-linked immunosorbent assay (ELISA)

The HUVEC culture supernatants were collected, clarified, and the levels of MCP-1 were quantified using a commercial ELISA kit (R&D Systems) according to the manufacturer's directions.

2.3. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from HUVEC and RT-PCR was done as described previously [9]. The sequences of primers were 5′-CAGCCAGATGCAATCAATGC-3′, 5′-GTGGTCCATGGAATCCTGAA-3′ for MCP-1 (198 bp); 5′-GAGTCTTTCTCCAACGTGAGC-3′, 5′-ACCTGGTACTTGAGTTTCCCA-3′ for cyclooxygenase-1 (COX-1, 350 bp); 5′-TCAATGAGGAGACTTGCCTG-3′, 5′-GATGAGTTGTCATGTCCTGC-3′ for IL-6 (260 bp); 5′-TTCAAATGA-

*Corresponding author. Fax: (82)-62-530 4855.
E-mail address: ickang@chonnam.ac.kr (I.-C. Kang).

0014-5793/04/$30.00 © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/S0014-5793(04)00058-4

GATTGTGGGAAAATTGCT-3′, 5′-AGTTCATCTCTGCCTGAG-TATCTT-3′ for COX-2 (305 bp); and 5′-AGCGGGAAATC-GTGCGTG-3′, 5′-CAGGGTACATGGTGGTGCC-3′ for β-actin (300 bp).

2.4. Western blot for Akt

HUVEC in 6-well plates were scraped with 100 μl of 1×Laemmli sample buffer (4% sodium dodecyl sulfate (SDS), 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, and 0.125 M Tris–HCl, pH 6.8). 20 μl of each boiled sample was resolved by SDS–polyacrylamide gel electrophoresis (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with a 1:1000 dilution of rabbit anti-phospho-Akt polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA) and a 1:1500 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology). Immunoreactive proteins were detected by enhanced chemiluminescence (Lumi-GLO, Cell Signaling Technology). The same membrane was stripped and reprobed with a 1:1000 dilution of anti-Akt (Cell Signaling Technology).

2.5. Gel shift assay for nuclear factor-κB (NF-κB)

Nuclear extracts were prepared from HUVEC according to the method of Dignam et al. [10]. Protein concentrations of the nuclear extracts were measured with DC Protein Assay Kit (Bio-Rad). Double-stranded NF-κB-binding DNA probe (5′-AGTTGAGGG-GACTTTCCCAGGC-3′) was purchased from Promega (Madison, WI, USA), and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. 15 μg of nuclear extracts were incubated with 10 000 cpm of probe in 20 μl of reaction buffer containing 10 mM Tris–HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, and 200 ng of poly(dI·dC) for 30 min at room temperature. Specific binding was controlled by competition with a 50-fold excess of cold NF-κB probe or irrelevant cAMP response element-binding protein (CREB) probe (Promega). The DNA-bound NF-κB proteins were separated on 4% polyacrylamide gels. The gels were dried and subjected to autoradiography.

3. Results and discussion

3.1. Activity of PI3K inhibitors

In order to confirm the PI3K-inhibiting activity of LY29 and WM at concentrations used in the present study, Akt phosphorylation was examined by Western blotting. Akt is phosphorylated by PI3K-generated phosphatidylinositol 3,4,5-trisphosphate and is thus sensitive to inhibition by PI3K inhibitors [11]. As shown in Fig. 1, both 50 μM LY29 and 100 nM WM completely inhibited IL-1β-stimulated Akt phosphorylation. At 50 μM, LY30, a compound structurally related to LY29 but known to have no PI3K-inhibitory activity [2], did not inhibit IL-1β-stimulated Akt phosphorylation. These data confirmed the ability of LY29 and WM to inhibit PI3K activity and showed that LY30 does not inhibit PI3K activity in our IL-1β-stimulated HUVEC model.

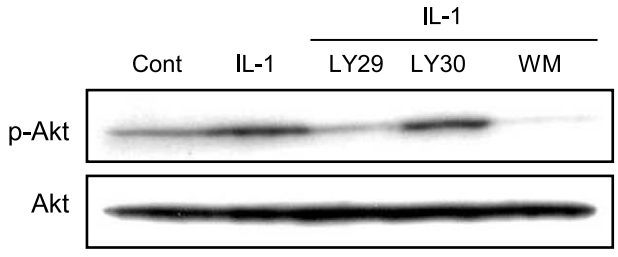


Fig. 1. Akt phosphorylation of IL-1β-, LY29-, WM-, or LY30-treated HUVEC. 3×10^5 HUVEC were seeded in 6-well plates. After 2 days, the cells were pretreated with LY29 (50 μM), LY30 (50 μM), or WM (100 nM) for 30 min prior to stimulation with IL-1β (10 ng/ml) for 30 min. Cell lysates were analyzed for Akt activation by Western blot analysis of total and phosphorylated Akt using specific antibodies. One of two experiments with similar results is shown.

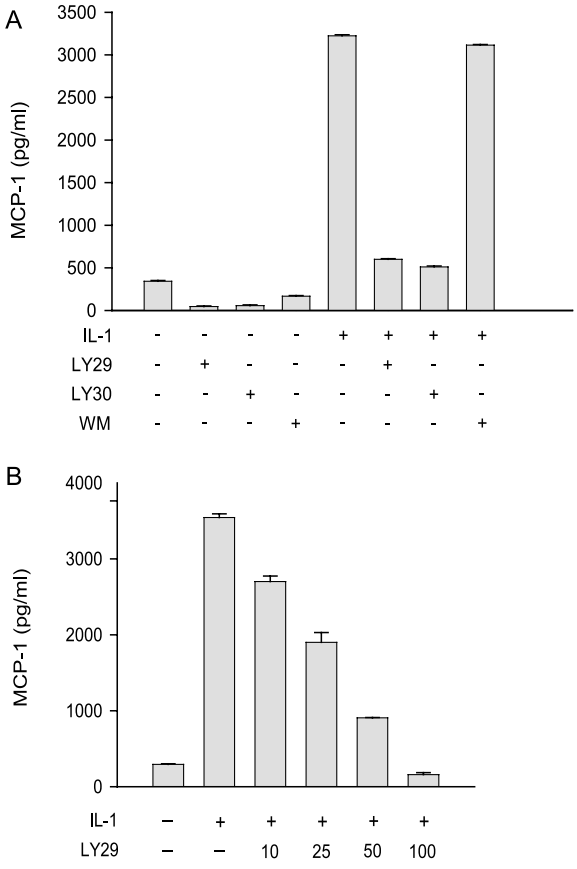


Fig. 2. MCP-1 production by HUVEC treated with IL-1β, LY29, WM, or LY30. A: 6×10^4 HUVEC were seeded in 48-well plates. After 1 day, the cells were pretreated with LY29 (50 μM), LY30 (50 μM), or WM (100 nM) for 30 min prior to stimulation with IL-1β (5 ng/ml) in a final volume of 0.5 ml for 8 h. MCP-1 concentrations of the culture supernatants were measured by ELISA. B: Dose response of LY29 on MCP-1 production. HUVEC were pretreated with various doses of LY29 for 30 min prior to stimulation with IL-1β (5 ng/ml) in a final volume of 0.5 ml for 8 h. Data are the means ± S.D. of a representative experiment performed in triplicate. Similar results were obtained in two other experiments.

3.2. Effects of PI3K inhibitors on MCP-1 protein production

Modulation of MCP-1 protein production by LY29, LY30, or WM was measured by ELISA. The level of MCP-1 in HUVEC culture supernatants increased about 10-fold by IL-1β stimulation (5 ng/ml). Treatment of cells with WM at 100 nM, which completely blocks PI3K activity, resulted in no attenuation of IL-1β-stimulated MCP-1 production by HUVEC. In contrast, at 50 μM, LY29 decreased IL-1β-induced MCP-1 production by about 80% (Fig. 2A). Not only LY29, but also LY30 (50 μM) reduced MCP-1 production to a similar extent. They also inhibited the basal MCP-1 production by unstimulated cells. There has recently been a report that LY29 and LY30 act as voltage-gated potassium (Kv) channel blockers in the MIN6 insulinoma cell line [12]. This has been the only report so far describing a PI3K-independent action of both LY29 and LY30; however, a much higher dose of LY30 (500 μM) was needed to achieve comparable ~90% inhibition

of Kv currents than of LY29 (50 μM). LY29 was originally developed from quercetin in search of more specific PI3K inhibitors and LY30 differs from LY29 by a single atom substitution. Quercetin, a naturally occurring bioflavonoid, has been shown to function as an anti-oxidant and anti-inflammatory agent with little toxicity [13]. Quercetin inhibits IL-1-induced MCP-1 expression in glomerular cells [14]. Therefore, it is likely that the common structure of quercetin, LY29, and LY30 inhibits MCP-1 production through a similar mechanism. The inhibitory effect of LY29 was dose-dependent (Fig. 2B). Attenuation of MCP-1 production by LY29 could be observed following a dose as low as 10 μM. The inhibitory effect of LY29 and LY30 is not restricted to endothelial cells, as they also inhibit IL-1β-stimulated MCP-1 production in phorbol ester-differentiated THP-1 macrophage cells (unpublished data). Taken together, these results clearly showed that LY29 inhibits MCP-1 production by endothelial cells, independent of the PI3K-Akt pathway.

3.3. Effects of LY29 and LY30 on MCP-1 mRNA expression

To assess the inhibitory effects of LY29 and LY30 on MCP-1 production at the steady-state mRNA level, RT-PCR analysis was carried out. The results of RT-PCR analysis were consistent with those of the ELISA assays of MCP-1 protein levels (Fig. 3). LY29 and LY30, but not WM, inhibited constitutive and IL-1β-induced MCP-1 mRNA expression. To rule out the possibility that the effect of LY29 and

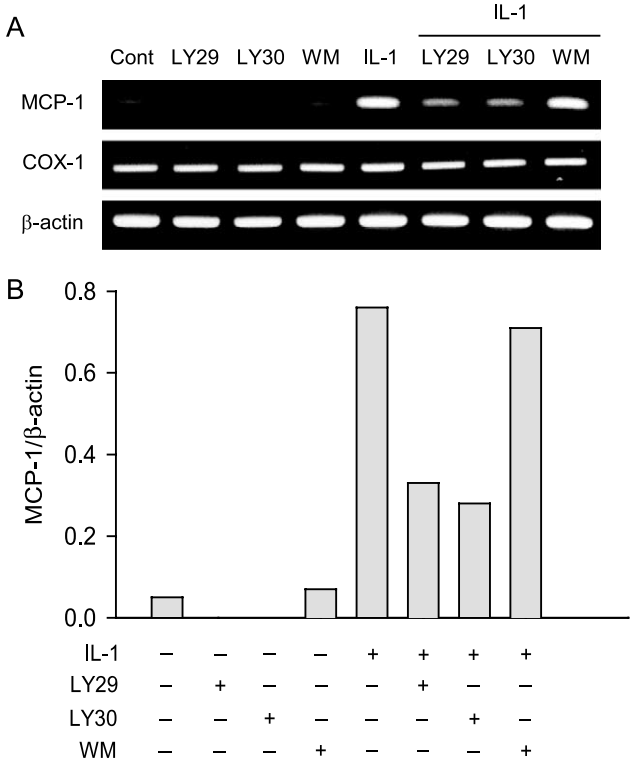


Fig. 3. MCP-1 and COX-1 mRNA expression by HUVEC treated with IL-1β, LY29, WM, or LY30. A: 3×10^5 HUVEC were seeded in 6-well plates. After 2 days, the cells were pretreated with LY29 (50 μM), LY30 (50 μM), or WM (100 nM) for 30 min prior to stimulation with IL-1β (5 ng/ml) for 4 h. Total RNA was extracted and expression of MCP-1 and COX-1 mRNA was determined by RT-PCR. B: Results are expressed as MCP-1/β-actin ratio by densitometric analysis. One of three experiments with similar results is shown.

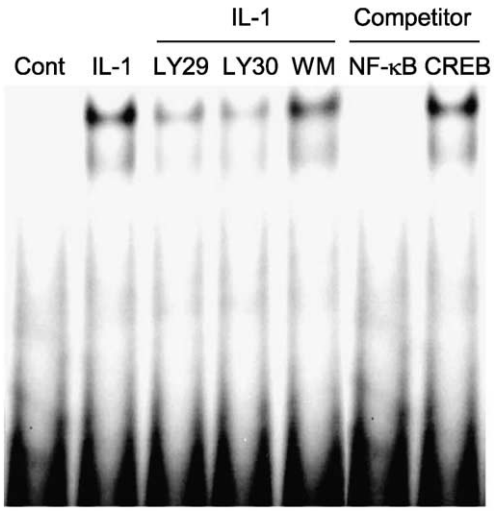


Fig. 4. NF-κB activation in HUVEC treated with IL-1β, LY29, WM, or LY30. 3×10^5 HUVEC were seeded in 6-well plates. After 2 days, the cells were pretreated with LY29 (50 μM), LY30 (50 μM), or WM (100 nM) for 30 min prior to stimulation with IL-1β (5 ng/ml) for 4 h. Nuclear extracts were prepared and NF-κB activation was determined by gel shift assays. NF-κB specificity was controlled with a 50-fold excess of cold NF-κB probe or irrelevant CREB probe. One of three experiments with similar results is shown.

LY30 may be attributable to their general inhibitory effect on cellular activities, COX-1 mRNA expression, known to be constitutive [15], was analyzed in parallel. COX-1 as well as β-actin mRNA expression was not affected by any treatment. These results indicated that LY29 and LY30 down-regulate MCP-1 production at the pre-translational level, and their action is not due to their general cellular inhibitory effects.

3.4. Effects of LY29 and LY30 on NF-κB activation

Since the MCP-1 inhibitory effect of LY29 and LY30 could be seen at the mRNA level and NF-κB is a critical transcription factor for MCP-1 gene expression [16], we performed gel shift assays to measure NF-κB activation. As shown in Fig. 4, the DNA binding activity of NF-κB induced by IL-1β was significantly inhibited by LY29 and LY30. The CREB probe and cold competition demonstrated that the bands are NF-κB-specific. This prompted us to determine whether the expression of other NF-κB-dependent genes is also inhibited by LY29 and LY30. As IL-6 and COX-2 genes, among IL-1-induced endothelial genes, contain NF-κB binding sites in their promoters and are controlled by NF-κB [17,18], we examined IL-6 and COX-2 mRNA expression by RT-PCR. Indeed, the IL-1β-induced mRNA expression of both IL-6 and COX-2 genes was significantly reduced by LY29 and LY30 (Fig. 5). These results are reminiscent of previous reports that quercetin has the ability to attenuate activation of NF-κB [14,19]. The molecular mechanisms for NF-κB suppression by quercetin have been suggested to be related to the fact that quercetin functions as an anti-oxidant or as a protein kinase inhibitor [14]. Quercetin-derived LY29 and LY30 may accordingly share the same mechanisms. Further studies are needed regarding how LY29 and LY30 inhibit NF-κB activation leading to attenuated MCP-1 production. Meanwhile, our data are consistent with a report that the PI3K/Akt pathway does not play a major role in the pro-inflamma-

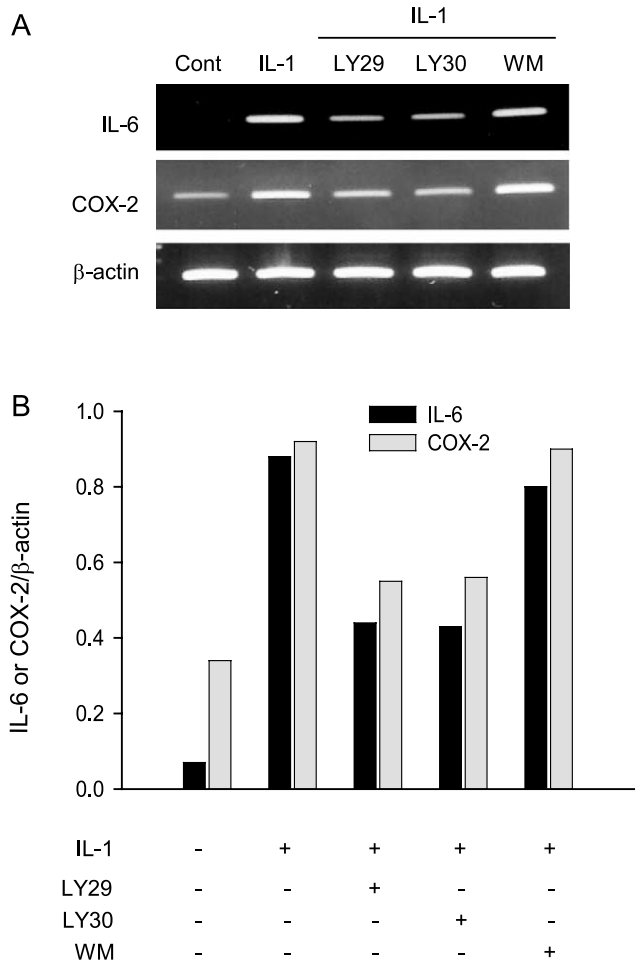


Fig. 5. IL-6 and COX-2 mRNA expression by HUVEC treated with IL-1β, LY29, WM, or LY30. A: 3×10^5 HUVEC were seeded in 6-well plates. After 2 days, the cells were pretreated with LY29 (50 μM), LY30 (50 μM), or WM (100 nM) for 30 min prior to stimulation with IL-1β (5 ng/ml) for 4 h. Total RNA was extracted and expression of IL-6 and COX-2 mRNA was determined by RT-PCR. B: Results are expressed as IL-6 or COX-2/β-actin ratio by densitometric analysis. One of three experiments with similar results is shown.

tory responses of endothelial cells to IL-1 [20]. Taken together, our results suggest that LY29 and LY30 inhibit MCP-1 expression at least in part via suppression of NF-κB, independent of PI3K. This represents the first report on the differential effects of LY29 and WM on cytokine production.

The present study gives two suggestions. First, it is strongly recommended that the effects of the two structurally different PI3K inhibitors WM and LY29 should be examined to reduce the risk that the observed effects of either PI3K inhibitor do not result from inhibition of PI3K. Second, further studies on the mechanisms of LY29 and LY30 mediating inhibition of NF-κB activation and MCP-1 expression may shed more light on pro-inflammatory signaling pathways, and the structure of quercetin-derived LY29 and LY30 may be a novel template for development of new anti-inflammatory drugs.

Acknowledgements: This study was supported by a research grant from Chonnam National University (I.C.K.).

References

[1] Adi, S., Wu, N.-Y. and Rosenthal, S.M. (2001) Endocrinology 142, 498–501.
[2] Vlahos, C.J., Matter, W.F., Hui, K.Y. and Brown, R.F. (1994) J. Biol. Chem. 269, 5241–5248.
[3] Arcaro, A. and Wymann, M.P. (1993) Biochem. J. 296, 297–301.
[4] Alberta, J.A., Auger, K.R., Batt, D., Iannarelli, P., Hwang, G., Elliott, H.L., Duke, R., Roberts, T.M. and Stiles, C.D. (1999) J. Biol. Chem. 274, 31062–31067.
[5] Walker, E.H., Pacold, M.E., Perisic, O., Stephens, L., Hawkins, P.T., Wymann, M.P. and Williams, R.L. (2000) Mol. Cell 6, 909–919.
[6] Salh, B., Wagey, R., Marotta, A., Tao, J.S. and Pelech, S. (1998) J. Immunol. 161, 6947–6954.
[7] Scheid, M.P. and Duronio, V. (1996) J. Biol. Chem. 271, 18134–18139.
[8] Gosling, J., Slaymaker, S., Gu, L., Tseng, S., Zlot, C.H., Young, S.G., Rollins, B.J. and Charo, I.F. (1999) J. Clin. Invest. 103, 773–778.
[9] Kang, I.-C. and Kuramitsu, H.K. (2002) FEMS Immunol. Med. Microbiol. 34, 311–317.
[10] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[11] Vivanco, I. and Sawyers, C.L. (2002) Nat. Rev. Cancer 2, 489–501.
[12] El-Kholy, W., Macdonald, P.E., Lin, J.H., Wang, J., Fox, J.M., Light, P.E., Wang, Q., Tsushima, R.G. and Wheeler, M.B. (2003) FASEB J. 17, 720–722.
[13] Formica, J.V. and Regelson, W. (1995) Food Chem. Toxicol. 33, 1061–1080.
[14] Ishikawa, Y., Sugiyama, H., Stylianou, E. and Kitamura, M. (1999) J. Am. Soc. Nephrol. 10, 2290–2296.
[15] Caughey, G.E., Cleland, L.G., Penglis, P.S., Gamble, J.R. and James, M.J. (2001) J. Immunol. 167, 2831–2838.
[16] Martin, T., Cardarelli, P.M., Parry, G.C., Felts, K.A. and Cobb, R.R. (1997) Eur. J. Immunol. 27, 1091–1097.
[17] Munoz, C., Pascual-Salcedo, D., Castellanos, M.C., Alfranca, A., Aragones, J., Vara, A., Redondo, J.M. and de Landazuri, M.O. (1996) Blood 88, 3482–3490.
[18] Schmedtje, J.F.Jr., Ji, Y.-S., Liu, W.-L., DuBois, R.N. and Runge, M.S. (1997) J. Biol. Chem. 272, 601–608.
[19] Cho, S.-Y., Park, S.-J., Kwon, M.-J., Jeong, T.-S., Bok, S.-H., Choi, W.-Y., Jeong, W.-I., Ryu, S.-Y., Do, S.-H., Lee, C.-S., Song, J.-C. and Jeong, K.-S. (2003) Mol. Cell. Biochem. 243, 153–160.
[20] Madge, L.A. and Pober, J.S. (2000) J. Biol. Chem. 275, 15458–15465.