Mitogen crosstalk accompanying urokinase receptor expression in stimulated vascular smooth muscle cells

Ute Reuning[a,*], Eric P. Dixon[b], Sheila P. Little[b], Nils U. Bang[c]

[a]*Frauenklinik der Technischen Universität München, Klinikum rechts der Isar, Ismaninger Str. 22, 81675 München, Germany*
[b]*Lilly Research Laboratories, Division of Cardiology and CNS Research, Eli Lilly and Co., Indianapolis, IN 46285, USA*
[c]*Indiana University School of Medicine, Department of Medicine and Pathology, Indianapolis, IN 46285, USA*

Received 17 April 1996; revised version received 3 July 1996

Abstract Thrombin and other mitogens regulate the expression of the urokinase-type plasminogen activator receptor (uPAR) protein and mRNA levels in bovine vascular smooth muscle cells (SMC). We investigated interactions between mitogens capable of increasing uPAR mRNA levels in SMC. Up-regulation of uPAR mRNA upon thrombin and basic fibroblast growth factor (bFGF) stimulation was preceded by a 2–3-fold transient increase in bFGF mRNA within 1 h. TGF-β_1 did not result in a significant change in bFGF mRNA levels. Platelet-derived growth factor (PDGF) while substantially enhancing uPAR mRNA levels, diminished bFGF mRNA levels by 3–4-fold. Both thrombin and bFGF induced the message for bFGF-R 2–3-fold. Thrombin also provoked a 3–4-fold rise in TGF-β_1 mRNA levels within 30 min. In summary, on the mRNA level, we demonstrated both positive as well as negative feed-back mechanisms between different mitogens, among them bFGF revealing in addition to autoinduction also up-regulation of the transcript concentration of its own receptor. Thus, cooperation and possible amplification of mitogenic effects might be implicated in the fine-tuned regulation of uPAR mRNA in stimulated bovine aorta SMC.

Key words: Atherosclerosis; Smooth muscle cell; Urokinase receptor; Mitogen

1. Introduction

Proliferation and migration of transformed secretory vascular smooth muscle cells (SMC) during atherosclerotic events are believed to be under the control of several mitogens. These mitogens not only increase new DNA synthesis and cell number of target cells [1], but may also promote cell invasiveness by several mechanisms, including the modulation of cell surface uPA/plasmin-mediated proteolytic activity [2]. One of the key components of the tissue fibrinolytic enzyme system is the cell surface receptor for uPA (uPAR) which enhances plasmin generation by binding with high affinity to active uPA as well as pro-uPA. uPAR has been identified on several tumor cells, monocytoid U937 cells [3] and on cultured bovine aorta SMC [4]. Several mitogens including thrombin, bFGF, PDGF, and TGF-β_1 were shown to produce uPAR affinity changes and increased receptor expression [4] coinciding with increased uPAR mRNA levels on bovine vascular SMC [5]. Most of these mitogens are thought to produce their biological effects in cooperation with other mitogens dependent on their interactions with specific cell surface receptors. In this report we studied possible mitogen 'crosstalk' occurring in stimulated SMC and monitored in parallel changes in uPAR mRNA levels.

2. Materials and methods

2.1. Materials

Bovine α-thrombin, PDGF, TGF-β_1, bFGF, and all tissue culture reagents were obtained from vendors as outlined previously [4,5]. An in vitro transcription kit, pGEM vectors, and a RNA isolation kit were from Promega, Madison, WI. A kit for RNase protection assays was from Ambion, Austin, TX. [^{32}P]UTP was obtained from NEN Dupont, Boston, MA. A kit for first-strand cDNA synthesis was purchased from BRL, Gaithersburg, MD. A plasmid isolation kit was obtained from Quiagen, Chlotsworth, CA. Reagents for polymerase chain reaction (PCR) were from Perkin Elmer Cetus, Norwalk, CT. Synthesis and purification of PCR primers were performed at Northwestern University, Chicago, IL.

2.2. Cell culture

Bovine aorta SMC were isolated, cultured and growth-arrested prior to stimulation as described earlier [4,5]. Stimulation at 37°C was performed with 40 U/ml thrombin, 20 ng/ml PDGF, 10 ng/ml bFGF, or 10 ng/ml TGF-β_1, concentrations chosen based on dose-response experiments on uPAR expression on SMC.

2.3. RNA isolation

Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction [6].

2.4. Isolation of cDNA clones and subcloning

A full-length cDNA clone for bovine uPAR was isolated from a bovine aorta endothelial cell cDNA library [7]. The generation of the bovine uPAR- and amyloid precursor protein (APP) cRNA has been described earlier [5]. The cDNA fragments for bovine bFGF (390 bp) and bovine TGF-β_1 (320 bp) were generated by PCR on bovine cDNA reverse-transcribed from total RNA, utilizing as primers *bFGF 5′*: GAG GAC GGC GGC AGC GGC GCT/*bFGF 3′*: CTG CCC AGG TCC TGT TTT GGG [8] and *TGF-β1 5′*: ACC AAC TAC TGC TTC AGC TCC ACA GAA/*TGF-β1 3′*: TTG CAG GAG CGC ACG ATC ATG [9], respectively, and subcloned into the transcription vector pGEM-4Z. Since the sequence for bovine bFGF-R is unknown, we designed degenerate PCR primers based on sequences for bFGF-R of known species counterparts [10]: *bFGF-R 5′*: CCG GAA TTC GAG/A GGC TGC TTT GGG CA and *bFGF-R 3′*: CGC GGA TCC GCC AGG TCT CT/GG TGT/G ATG GA. The nucleotide sequences of the PCR-generated DNA fragments were confirmed by sequencing analyses.

2.5. In vitro transcription and RNase protection assay (RPA)

In vitro transcription and RPA were performed as described earlier [5]. Intensity of the signals on autoradiographs of dried gels was quantitatively analyzed by laser densitometry (Ultroscan XL Laser Densitometer, Pharmacia, Piscataway, NJ). The relative abundance of uPAR, bFGF-, bFGF-R-, and TGF-β_1 mRNA was determined by normalizing the integrated signal from the resulting protected fragments to the integrated signal of the internal standard APP probe. Alternatively, original dried gels were scanned in a β-scanner (Betascope 603 Blot Analyzer, Betagen, Mountain View, CA). The relative amounts are given as 'fold increase'/unstimulated control. All given values were obtained from three independent repeats of experiments.

*Corresponding author. Fax: (49) 89-4140-4846.

PII S0014-5793(96)00797-1

Message levels in total RNA from unstimulated cultured SMC co-hybridized with the cRNA probes used in this study did not change significantly during the same time period. Upon stimulation of SMC with different mitogens we did not observe significant changes in the signals obtained from the internal standard APP.

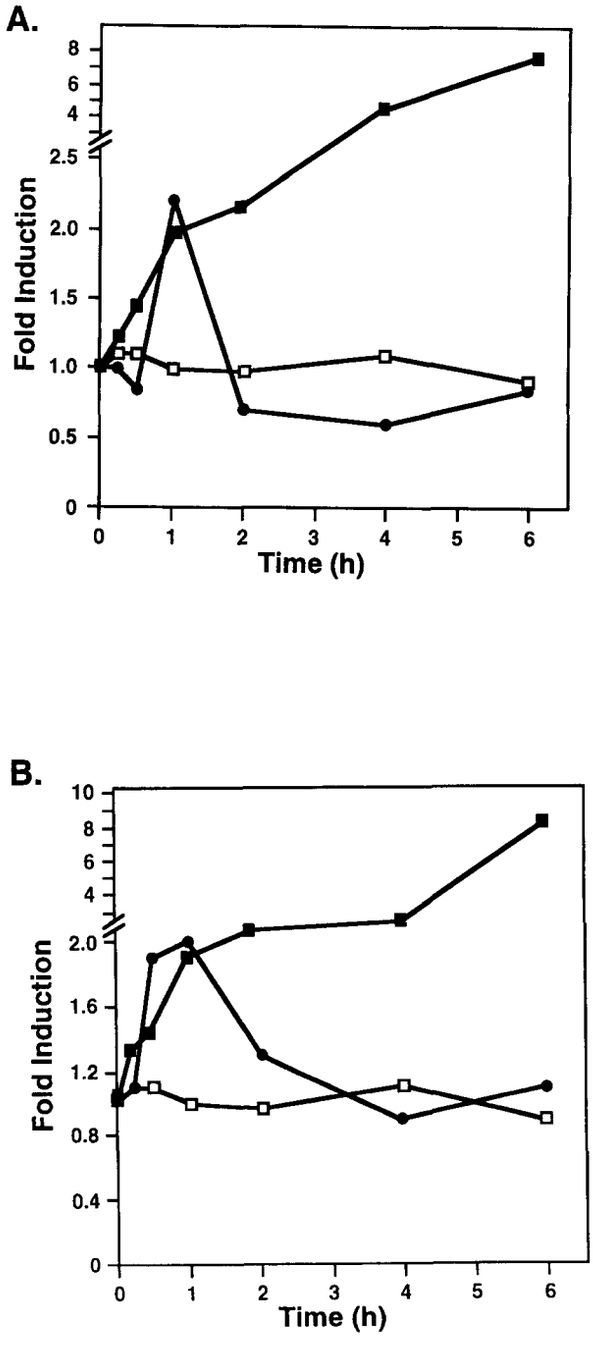

Fig. 1. Time-dependent effect of thrombin and bFGF on bFGF- and uPAR mRNA levels in bovine vascular SMC. A: SMC were exposed to thrombin (40 U/ml), total RNA isolated and RPA performed. The relative abundance of mRNA for bFGF (●), uPAR (■) and APP (□) measured and calculated by laser densitometry are outlined in Section 2. The values at each time point are given as 'fold induction' assigning a value of '1' to the message level at time '0'. Each value represents the mean of triplicate experiments. B: SMC exposed to bFGF. Methods as described above. bFGF (●), uPAR (■), and APP (□).

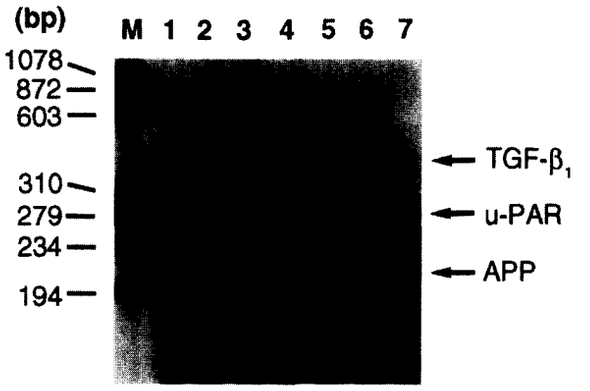

Fig. 2. Effect of thrombin on TGF-β_1- and uPAR mRNA levels in vascular SMC. Bovine SMC were exposed to thrombin (40 U/ml) and harvested at time point '0' (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), and 6 h (lane 7). ^{32}P-labeled TGF-β_1-, uPAR-, and APP cRNAs were co-hybridized with 10 μg of total SMC RNA overnight at 42°C. RPA were performed as described and the protected fragments (TGF-β_1: 320 bp, uPAR: 279 bp; APP: 229 bp) electrophoresed on Tris-borate-EDTA/polyacrylamide/urea gels which were dried and exposed to X-ray films for autoradiography. The radiolabeled TGF-β_1, uPAR, and APP cRNA probes are shown in lane 12. M: ^{32}P-labeled Lambda *Hin*dIII, PhiX *Hae*III marker.

3. Results and discussion

There is evidence that cell invasiveness requires cell surface proteolytic activity, e.g., plasmin, capable of altering cell–cell and cell–substratum interactions. The existence of uPAR on vascular SMC in culture [4], which strongly resemble the transformed SMC phenotype, suggested that uPAR might be involved in SMC migration during atherosclerotic events. Mitogens available at the site of atherosclerotic lesions, originating from platelets, monocytes/macrophages, endothelial cells, and neointimal SMC, all cells that are present within the atheromatous plaque, are known to regulate uPAR expression [11]. At sites of fracture of atheromatous plaques, platelets accumulate setting the stage for thrombin generation which can initiate and propagate an intravascular thrombus and also act as a mitogen for adjacent cells in the vessel wall. Thrombin in addition to other growth factors may not work separately in the propagation of atheromatous plaque formation but may act cooperatively with other growth factors through additive, synergistic or 'cascade-like' effects [12].

In the present study on bovine vascular SMC we examined elevation of mRNA levels for different mitogens upon stimulation while monitoring in parallel increases in uPAR mRNA levels. Exposure of SMC to thrombin previously shown to increase uPAR mRNA levels [4] resulted in a transient 2–3-fold increase in bFGF message levels. Peak levels at approximately 30 min with a rapid decline to basal levels preceded the rise in uPAR mRNA at 4 h (Fig. 1). This is in agreement with observations that thrombin provoked the appearance of bFGF detected in cellular lysates of vascular SMC [13] and fibroblasts [14], suggesting a role for bFGF in thrombin's mitogenic activity. It has been reported earlier that both thrombin but also TGF-β_1 enhance the expression of other growth factors (e.g., PDGF) in human microvascular endothelial cells and vascular SMC followed by an increase in growth-factor-like activity in conditioned media [15,16]. In addition, we also observed that thrombin induced TGF-β_1

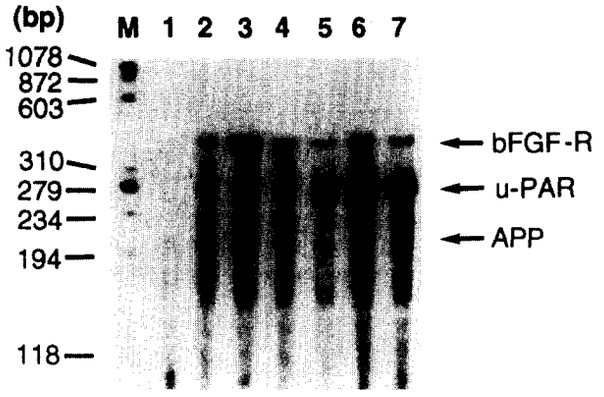

Fig. 3. Time-dependent effect of bFGF on bFGF-R mRNA levels. Bovine aorta SMC were exposed to bFGF (10 ng/ml). The cells were harvested at time point '0' (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), and 6 h (lane 7). RNA was hybridized with ^{32}P-labeled bFGF-R-, uPAR-, and APP cRNAs and RPA performed. The relative abundance of the bFGF-R (417 bp) and uPAR protected fragment (279 bp) were determined by scanning the gels in a β-scanner, normalizing the cpm values to the signals of interest to the signal from the internal standard APP. M: ^{32}P-labeled Lambda *Hin*dIII, PhiX *Hae*III marker.

mRNA approximately 3–4-fold within 30 min followed by a return to basal levels within approximately 4 h (Fig. 2).

It is known that several growth factors are capable of inducing their own message expression in vitro. [17,18]. Indeed, bFGF produced a 2–3-fold increase in its own transcript concentration (Fig. 1). Basic FGF is widely distributed and seems to be implicated in cell proliferation and cell migration in several different pathologies including tumor growth and atherosclerosis [19,20]. During remodeling of the matrix, bFGF is mobilized as a complex with heparan sulphate proteoglycans by the action of cell surface plasmin and subsequently binds to specific cell surface receptors [21]. The bioavailability of bFGF is thus directly under the control of the uPA/plasmin-mediated proteolytic activity on cell surfaces [22]. This might suggest positive feedback mechanisms: enhanced plasmin generation facilitated by increased uPAR numbers upon bFGF stimulation promotes the release of bFGF from the extracellular matrix [23]. Moreover, bFGF is a potent activator of SMC proliferation [24]. In contrast to bFGF, TGF-β_1, which significantly increased uPAR message levels [5], did not reveal any effect on its own message (data not shown), an observation in contrast to other reports describing TGF-β_1 autoinduction [25].

Increased responsiveness of transformed migratory SMC and perhaps other cell types to elevated mitogen levels necessitates the presence of sufficient numbers of available specific mitogen receptor sites. On cultured vascular SMC we observed that both thrombin and bFGF increased bFGF-R mRNA levels 2–3-fold coinciding with increased bFGF mRNA. This rise preceded the increase in uPAR mRNA levels, initially detectable at time point 4 h (Fig. 3). This is in agreement with the observation that enhanced bFGF and bFGF-R expression also occurs in invasive carcinoma cell lines but not in non-invasive cells [26]. Moreover, thrombin as well as bFGF increased mRNA levels of the thrombin receptor [5] strongly implicated in thrombin-mediated cellular effects on platelets and endothelial cells.

In PDGF stimulated SMC an unexpected decline in bFGF mRNA was observed. A 3–4-fold decrease, initially detectable at 4 h, coincided with the rise in uPAR mRNA levels (Fig. 4). Although several examples of a mutual down-regulation among mitogens are published, including the decrease of bFGF's mitogenic activity by TGF-β_1 [27] or the down-regulation of TGF-β_1 binding to its receptor by bFGF [14], the relevance of the down-regulation of bFGF mRNA levels in SMC by higher concentrations of PDGF are unclear at this time. In principle, it should be noted here critically that increases in mRNA concentrations of certain proteins do not necessarily reflect respective changes in protein expression even in general this has been shown to be the case.

The observed induction of mitogen mRNA expression in cultured bovine vascular SMC, data which although not necessarily reflecting similar changes on the protein levels of the respective mitogens, are, however, very much consistent with

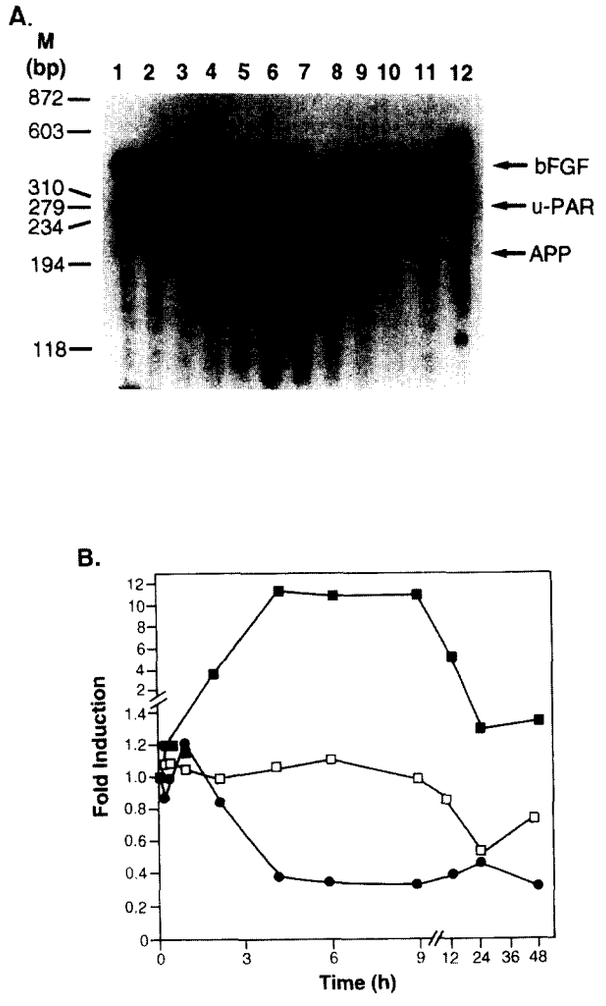

Fig. 4. Time course of the effect of PDGF on bFGF and uPAR mRNA levels in bovine vascular SMC. A: SMC were exposed to PDGF-BB (20 ng/ml) and the cells harvested at time point '0' (lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 6 h (lane 7), 9 h (lane 8), 12 h (lane 9), 24 h (lane 10), and 48 h (lane 11). cRNA probes for bFGF, uPAR as well as APP were co-hybridized and the protected fragments electrophoresed on Tris-borate-EDTA/polyacrylamide/urea gels which were dried and exposed to X-ray films for autoradiography. The radiolabeled bFGF-, uPAR-, and APP cRNAs are shown in lane 12. B: Value at each time point in Fig. 4A expressed as 'fold induction': bFGF (●), uPAR (■), APP (□).

in situ findings that transcripts for several growth factors and their receptors including PDGF-B, the β-subunit of the PDGF receptor, and TGF-β_1 were increased in advanced atherosclerotic lesions. In contrast, growth-promoting molecules and their specific receptors are not expressed in SMC from the tunica media of normal arteries or in regions of the intima unaffected by atherosclerosis [28].

References

[1] Moses, H.L., Yang, E.Y. and Pietenpol, J.A. (1990) Cell 63, 245–247.
[2] Laiho, M. and Keski-Oja, J. (1989) Cancer Res. 49, 2533–2553.
[3] Conese, M. and Blasi, F. (1995) Biol. Chem. Hoppe-Seyler 376, 143–155.
[4] Reuning, U. and Bang, N.U. (1992) Arterioscler. Thromb. 12, 1161–1170.
[5] Reuning, U., Little, S.P., Dixon, E.P. and Bang, N.U. (1994) Blood 84, 3700–3708.
[6] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[7] Reuning, U., Little, S.P., Dixon, E.P., Johnstone, E.M. and Bang, N.U. (1993) Thromb. Res. 72, 59–70.
[8] Abraham, J.A., Mergia, A., Whang, J.L., Tumolo, A., Friedman, J., Hjerrild, K. A., Gospodarowicz, D. and Fiddes, J.C. (1986) Science 233, 545–548.
[9] Van Obberghen-Schilling, E., Kondaiah, P., Ludwig, R.L., Sporn, M.B. and Baker, C.C. (1987) Mol. Endocrinol. 1, 693–698.
[10] Johnson, D.E., Lee, P.L., Lu, J. and Williams, L.T. (1990) Mol. Cell Biol. 10, 4728–4736.
[11] Ross, R. (1993) Nature 362, 801–809.
[12] Leof, E.B., Proper, J.A., Goustin, A.S., Shipley, G.D., DiCorletto, P.E. and Moses, H.L. (1986) Proc. Natl. Acad. Sci. USA 83, 2453–2457.
[13] Lowe, W.L., Yorek, M.A. and Teasdale, R.M. (1993) Endocrinology 132, 1593–1602.
[14] Fafeur, F., Terman, B.I., Blum, J. and Bohlen, P. (1990) Growth Factors 3, 237–245.
[15] Daniel, T.O., Gibbs, V.C., Milfay, D.F., Garovoy, M.R. and Williams, L.T. (1986) J. Biol. Chem. 261, 9579–9582.
[16] Kanthou, C., Parry, G., Wijelath, E., Kakkar, V.V. and Demoliou-Mason, C. (1992) FEBS Lett. 314, 143–148.
[17] Coffey, R.J., Derynck, R., Wilcox, J.N., Bringman, T.S., Goustin, S.A., Moses, H.L. and Pittelkow, M.R. (1987) Nature 328, 817–820.
[18] Paulsson, Y., Hammacher, A., Heldin, C.H. and Westermark, B. (1987) Nature 328, 715–717.
[19] Moscatelli, D. (1987) J. Cell Physiol. 131, 123–130.
[20] Partanen, J., Mäškelä, T.P., Eerola, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L. and Alitalo, K. (1991) EMBO J. 10, 1347–1354.
[21] Klagsbrun, M. and Baird, A. (1991) Cell 67, 229–231.
[22] Saksela, O. and Rifkin, D.B. (1990) J. Cell Biol. 110, 767–775.
[23] Vlodavsky, I., Folkman, J., Sullivan, R., Friedman, R., Ishai-Michaeli, R., Sasse, J. and Klagsbrun, M. (1987) Proc. Natl. Acad. Sci. USA 84, 2292–2296.
[24] Kraemer, R., Pomerantz, K.B., Joseph-Silverstein, J. and Hajjar, D.P. (1993) J. Biol. Chem. 268, 8040–8045.
[25] Van Obberghen-Schilling, E., Roche, N.S., Flanders, K.C., Sporn, M.B. and Roberts, A.B. (1988) J. Biol. Chem. 263, 7741–7746.
[26] Allen, L.E. and Maher, P.A. (1993) J. Cell Physiol. 155, 368–375.
[27] Frater, S.M., Muller, G., Birchmeier, W. and Bohlen, P. (1986) Schweiz. Med. Wochenschr. 116, 1160–1162.
[28] Nikol, S., Isner, J.M., Pickering, J.G., Kearney, M., Leclerc, G. and Weir, L. (1992) J. Clin. Invest. 90, 1582–1592.