Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 323-331

ADENYLYL CYCLASE ISOFORMS ARE DIFFERENTIALLY EXPRESSED IN PRIMARY CULTURES OF ENDOTHELIAL CELLS AND WHOLE TISSUE HOMOGENATES FROM VARIOUS RAT TISSUES

Vangelis G. Manolopoulos[1,2,*], Jiandong Liu[1,*], Brian R. Unsworth[2], and Peter I. Lelkes[1]

[1]Laboratory of Cell Biology, Department of Medicine, University of Wisconsin Medical School, Milwaukee Clinical Campus at Sinai-Samaritan Medical Center, Milwaukee, Wisconsin 53201

[2]Department of Biology, Marquette University, Milwaukee, Wisconsin 53201

Received January 23, 1995

The expression of five adenylyl cyclase isoforms (types II-VI) was studied with reverse transcription-polymerase chain reaction in whole tissue homogenates and in primary cultures of endothelial cells isolated from rat aorta, vena cava, heart, lung, adipose fat, brain, and adrenal medulla. It was found that: i) all endothelial cell types express all adenylyl cyclase isoforms studied, albeit at different levels depending on the tissue origin of the cells, and ii) the adenylyl cyclase isoform profile of isolated endothelial cells differs from that of homogenates of their parent tissues. Our data show a unique endothelial cell type specificity of AC isoform expression, which varies from that of the whole organ. These results support the idea that one of the factors mediating differential regulation of the cAMP cascade in EC in various locations within the vascular tree might be quantitative differences in the levels of AC isoforms expressed in each EC type.

Adenylyl cyclase (AC) catalyzes the formation of the ubiquitous second messenger cAMP which participates in the regulation of multiple mammalian cell functions, including growth, proliferation, differentiation, secretion, and shape changes (1). Molecular cloning studies have revealed that the AC family comprises at least 8 different isoforms (2,3). Full-length cDNAs for seven of these isoforms have been cloned (4-10), including rat-specific sequences for six of them (types II-VI, VIII). Some information is available on the tissue distribution of individual AC isoforms: Type I appears to be neural-specific; its restricted

* These authors contributed equally to this work.

Abbreviations used: EC, endothelial cells; MEC, microvascular endothelial cells; AC, adenylyl cyclase; RT, reverse transcription; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; ECGS, endothelial cell growth supplement; dil-ac-LDL, acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-1,3,3,3',3'tetramethylindocarbo-cyanine-perchlorate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

0006-291X/95 $5.00

expression to certain areas of the brain suggests a possible role in neuroplasticity (10,11). Type III, originally thought to be exclusively expressed in olfactory epithelium (6), exhibits a broad tissue distribution (12). Type V, originally isolated from the heart (8), was later also found, together with the related isoform VI, in several other tissues, most prominently brain (9,13-15). Type II has so far been found in brain and lung (5,7). Finally, type IV has been detected in a variety of tissues, most prominently liver, heart, and kidney (7). It should be noted, however, that most of these findings were derived by analysis of whole tissue extracts. The usefulness of such studies is limited, as each tissue is composed of multiple cell types, each of them performing specialized functions. Therefore, only studies on AC isoforms expressed in individual cell types will yield meaningful correlations between particular AC isoforms and specific cellular function(s).

Vascular endothelial cells (EC) in distinct anatomical sites exhibit profound phenotypic and functional differences (16-18). We have recently shown that the cAMP cascade in various types of EC is differentially regulated by extracellular stimuli, such as forskolin, isoproterenol, histamine, and by cyclic mechanical strain (19,20). These studies unveiled AC as one of the possible sites where this differential regulation might occur and prompted us to hypothesize that EC derived from distinct vascular beds might express different AC isoforms (20). In order to test this hypothesis, we prepared primary EC cultures from several rat tissues and assayed them for the presence of five AC isoforms (types II-VI) using a sensitive reverse transcription-polymerase chain reaction (RT-PCR) assay. In addition, we assessed how the AC isoform expression profile of each EC type correlates with that of the corresponding whole tissue, by measuring mRNA message for each of the same AC isoforms in whole tissue homogenates. We found significant differences in the levels of AC isoform expression among the various EC types and, even more significantly, between each EC type and its respective tissue of origin. Our results suggest that the expression profile of AC isoforms in any given EC type is highly specific and not immediately apparent from the corresponding whole tissue isoform profile.

MATERIALS AND METHODS

All reagents were from Sigma Chemical Co. unless otherwise specified in the text.

EC Isolation and Culture: Adult Sprague-Dawley rats were sacrificed and the following tissues quickly removed: brain, lung, adrenal medulla, adipose fat, heart, aorta and vena cava. The tissues were either minced and directly subjected to total RNA extraction or used for EC isolation, as described below.

Microvascular endothelial cells (MEC) from brain, lung, adrenal medulla, and adipose fat were isolated with a modification of an established method (21), as previously described (19,20). Briefly, the tissues were minced, digested with 0.2% collagenase, centrifuged, and sequentially filtered through 150 μm, 70 μm and 20 μm pore nylon meshes. Capillary

fragments retained on the 70 μm and 20 μm filters were grown to confluence in 6-well plates with M199 medium (Mediatech Inc.) supplemented with 10% (v/v) FCS (HyClone), 30 μg/ml ECGS, 50 μg/ml heparin, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and 5 μg/ml fungizone.

Microvascular EC from heart were isolated with a modification of the method recently described by Nishida et al (22). Briefly, connective and valvular tissue was removed, and the heart was immersed in 70% ethanol for 30 sec in order to devitalize epicardial mesothelial cells and endocardial EC. After extensive washing, the tissue was minced exhaustively in 0.2% collagenase in Ca^{2+}-free PBS and incubated for 30 min at 37°C in a shaking water bath. Trypsin (0.02%) was added and the tissue was sheared 10 times through a 18G needle and incubated for another 30 min. Following sequential filtration as described above, cells retained on the 70 μm and 20 μm meshes were grown to confluence in 6-well plates with DMEM (Mediatech Inc.) supplemented with 10% FCS, 30 μg/ml ECGS, 50 μg/ml heparin, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml fungizone.

Large vessel EC from the aorta and the vena cava were isolated with a modification of an established method (23). Briefly, the full length aorta or abdominal vena cava was removed from the rat, cleaned from adhering fat and connective tissue, and cut into small pieces (0.5 cm x 0.5 cm). The pieces were transferred onto gelatin-coated 12-well plates [endothelial layer side down] and allowed to attach to the substrate for 5 hours, in a humidified incubator at 37°C in a 95% air/ 5% CO_2. This initial incubation period with small volume of medium was important to ensure that the explant adhered to the substratum. Thereafter, an additional small volume of DMEM (supplemented as above) was added and the incubation continued for 48 hours. The explant was then removed and the attached EC were grown to confluence.

The purity and endothelial cell nature of all primary cultures was assessed by monitoring the capacity of the cells to take up acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-1,3,3,3',3'tetramethylindocarbo-cyanine-perchlorate (dil-ac-LDL, Biomedical Technologies Inc.), following a previously described protocol (24). At the time of RNA extraction, more than 95% of the cells in the confluent or nearly confluent EC monolayers were positive for dil-ac-LDL. Also, cells were used within a week after isolation and had undergone less than 6 population doublings, thus minimizing the possibility of culture-induced de-differentiation.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR): The protocols used in our laboratory for RT-PCR have recently been described (25). Briefly, total RNA was prepared from EC monolayers and tissue homogenates by the single-step guanidinium thiocyanate-phenol-chloroform extraction method using a commercially available kit (RNA STAT-60, from Tel-Test "B" Inc.), according to the manufacturer's instructions. RNA was reverse transcribed, using oligo-dT as the primer and 10 U/μl Moloney murine leukemia virus reverse transcriptase (USB Corp.), at 37°C for 2 hours. For the PCR amplification step we designed PCR primer pairs for AC isoforms II-VI and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from published rat-specific sequences (5-9, 26). All primers were designed in such a way that the 5' primer and 3' primer were located in two adjacent exons. All primer sequences and their respective sources are shown in Table I. The PCR amplification was performed according to standard protocols. PCR products were electrophoresed in agarose gels, stained with ethidium bromide, and photographed under UV light. Verification of the bands was done by comparing with the predicted band size and by the fact that only a single band was obtained in each case.

The image of the PCR products on the gel was digitized on a Complete Page Scanner and the intensity of the bands was analyzed using the QGEL program (Kendrick Labs), as described elsewhere (25). The relative level of expression of each isoform was determined by calculating the ratio of the intensity of an isoform band to that of the GAPDH band in the same RNA preparation. Differences in expression of isoforms in different cultures or

Table I: Adenylyl Cyclase isoform and GAPDH oligonucleotide primer sequences

Gene	Primer	Sequence	Ref. #
AC II	sense	5'-CGTGTCACTCTCCATATTC-3'	(5)
	antisense	5'-CCTTGTTCACATCTGACTC-3'	
AC III	sense	5'-CATCGAGTGTCTACGCTTC-3'	(6)
	antisense	5'-GGATGACCTGTGTCTCTTCT-3'	
AC IV	sense	5'-TTCTTCACACTCCTCGTCC-3'	(7)
	antisense	5'-CGTCCTGTTGTGTGTCCTG-3'	
AC V	sense	5'-ATCGAGCTCATCTACGTGC-3'	(8)
	antisense	5'-AGCATGCAGATACAGAGCC-3'	
AC VI	sense	5'-CTGCTTGTGTTCATCTCTG-3'	(9)
	antisense	5'-GACGCTAAGCAGTAGATCA-3'	
GAPDH	sense	5'-GTGCTGAGTATGTCGTGGA-3'	(26)
	antisense	5'-CACAGTCTTCTGAGTGGCA-3'	

All primers were designed according to the rat-specific sequences published in the references indicated.

tissues were determined by comparing those ratios. As the intensity of the GAPDH band was approximately 100 times higher than the intensity of any of the AC isoform bands, comparisons were done to 100-fold dilutions of GAPDH. Similar unique tissue/cell specific banding patterns for all five isoforms investigated were obtained in at least three independent runs.

RESULTS AND DISCUSSION

Typical profiles of AC isoform expression are exemplified in Figure 1 for adipose MEC and lung MEC. All PCR reactions yielded single-banded products of the anticipated size. After digitization, the intensities of these bands were normalized to that of GAPDH (Figure 1, lane 1), as described in methods. The validity of GAPDH as housekeeping gene in EC has been questioned by the recent report that it behaves as an early response gene in cultured human umbilical vein EC exposed to fresh serum-containing medium (27). However, in our case, GAPDH expression was fairly stable (variations < 20%) in all the tissues and EC types examined (data not shown).

Our results for five AC isoforms assessed in primary cultures of seven EC types are summarized in Table II. All isoforms were detected in all EC types. However, while mRNA message for isoforms III, V, and VI was rather uniformly abundant, message for isoforms

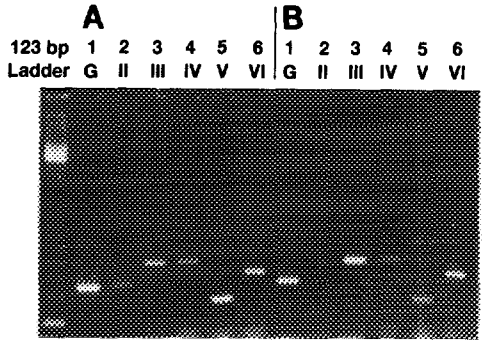

Figure 1. Adenylyl cyclase isoform expression in primary cultures of microvascular endothelial cells isolated from rat adipose fat (A) and lung (B). Total RNA was extracted from the cells, reverse transcribed, and PCR performed with isoform-specific primers as described in Materials and Methods. Lane 1: GAPDH; lane 2: AC type II; lane 3: AC type III; lane 4: AC type IV; lane 5: AC type V; lane 6: AC type VI.

Table II: Adenylyl cyclase isoform expression in primary cultures of endothelial cells (EC) and whole tissue homogenates (WT) from various rat tissues

AC isoform	II	III	IV	V	VI
Adrenal EC	tr	+++	tr	+	+++
Adrenal WT	-	tr	++++	+	+++
Brain EC	++	++++	+++	++	+++
Brain WT	+++	+++	-	++	tr
Adipose EC	++	++	+	+++	++
Adipose WT	-	+	-	tr	-
Lung EC	tr	++++	+	++	+++
Lung WT	++	+++	tr	++	++
Heart EC	+	+++	+++	+++	+++
Heart WT	-	tr	-	+++	++
Vena Cava EC	tr	+++	tr	+++	+++
Vena Cava WT	tr	++	-	tr	tr
Aorta EC	+	+++	tr	++	++
Aorta WT	+	++	-	tr	-

Total RNA was prepared from the indicated tissue, reverse transcribed, and used as a template for amplification with rat-specific primers, as described in Materials and Methods. The level of expression of each AC isoform was calculated by comparing the intensity of the band corresponding to that isoform with the intensity of the band corresponding to GAPDH from the same run. Results are averages of three independent runs with different batches of cells, and they are indicative of the ratio of the AC isoform intensity versus that of the GAPDH band, as follows: tr < 0.100, + ≥ 0.100, ++ ≥ 0.200, +++ ≥ 0.400, ++++ ≥ 0.750. Similar unique tissue/cell specific banding patterns for all five isoforms investigated were obtained in three independent runs.

II and IV exhibited significant variations between the various EC types: EC from adrenal medulla, aorta, and vena cava expressed only trace amounts of isoform IV message, while abundant message for that isoform was detected in EC from brain and heart (Table II). Also, only trace amounts of isoform II message were detected in EC from adrenal medulla, lung, and vena cava, in contrast to the abundant message found in brain and adipose fat EC.

In contrast to our findings in isolated EC, the whole tissue homogenates - with the exception of lung - expressed message for only some of the AC isoforms, even though we used a very sensitive technique (RT-PCR). (Table II). In the lung, message for all the AC isoforms studied was detected. Thus, in agreement with previous reports (5-8,10,11), our results with whole tissue homogenatess show restricted expression of specific AC isoforms to certain tissues.

Surprisingly, the AC isoform expression profile in the individual EC types did not match the profile in their respective tissues of origin. For example, in the whole heart, we found abundant message for isoforms V and VI, trace amounts of isoform III, and no evidence for the presence of isoforms II and IV (Figure 2A). In primary cultures of microvascular EC isolated from the same hearts, however, message for all AC isoforms was detected (figure 2B). Similarly, only trace amounts of message for isoform V and VI was detected in whole aorta and vena cava, while abundant message for both isoforms was found in primary EC cultures from the same vessels (Table II). Finally, in the adrenal, isoform IV is hardly, if at all, expressed in primary EC cultures, but strongly expressed in whole tissue.

These results should mainly be viewed as a qualitative indication of which types of AC are expressed in each tissue; their quantitative value is limited. And yet, the data clearly show that the AC isoform profile is highly cell- and tissue-specific. By comparing AC isoform expression in primary cultures of EC and whole tissue extracts, we conclude that there is substantial variation in the expression of AC isoforms in vascular EC and in the

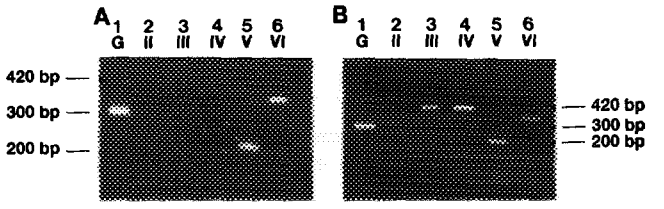

Figure 2. Adenylyl cyclase isoform expression in (A) rat heart homogenatess, and (B) primary cultures of rat heart microvascular endothelial cells (B). Total RNA was extracted from the cells, reverse transcribed, and PCR performed with isoform-specific primers as described in Materials and Methods. Lane 1: GAPDH; lane 2: AC type II; lane 3: AC type III; lane 4: AC type IV; lane 5: AC type V; lane 6: AC type VI.

other cell type(s) present in each tissue. We might speculate that in some instances (e.g. heart), our failure to detect certain AC isoforms (types II, IV) in whole tissue homogenatess (figure 2A) is simply a matter of concentration: If a particular cell type (such as MEC in cardiac tissue), that uniquely express a certain AC isoform is only a minority population in that tissue, extraordinary numbers of PCR cycles might be needed before a band in whole tissue extracts can be detected. On the other hand, in the adrenal medulla, EC and parenchymal cells each constitute approximately half of the total cell mass (28). Therefore, the concurrence of the data for isoforms II, V, and VI in whole tissue extracts and isolated endothelial cells is not surprising. Moreover, we can postulate that isoform IV is solely expressed in the parenchymal cells, while isoform III is specifically expressed in EC. *In situ* hybridization studies are currently under way to test this hypothesis.

The functional consequences of each cell type expressing more than one isoform remain to be analyzed. Each isoform has distinct properties and shows a unique mode of regulation, thus exhibiting the potential for differential roles within the cell. For example, while all isoforms can be activated by forskolin and the α subunit of G_s-protein, only isoforms I, II, and IV are regulated by G-protein $\beta\gamma$ subunits, either in an inhibitory (type I)(4) or in a stimulatory fashion (types II and IV)(5,7). It should be noted that the latter effect occurs only in the presence of activated $G_s\alpha$. Furthermore, isoforms I, III, and VIII are stimulated by Ca^{2+}/ calmodulin (4,6,10), while isoform VI is inhibited at low concentrations of free Ca^{2+}, without involvement of calmodulin (9). Also, the direct, isoform-specific interaction of AC with specific protein kinase C isoenzymes has been reported (29). One theory that has been proposed to explain this high degree of AC specialization is that particular AC isoforms may serve as unique sites where information from several signal transduction systems is integrated and translated into adjustment of the cAMP levels, in order to accurately serve the specific spatial and temporal needs of the cell (3).

Two recent studies in the heart link abnormalities in the expression of specific AC isoforms with pathophysiological states: In the first, a decrease of the AC isoform VI content correlated well with a decrease in catalytic activity of the myocardial β-adrenergic receptor-G_s-adenylyl cyclase pathway observed with age (30). Also, during pacing-induced heart failure in dogs, basal as well as forskolin-stimulated AC activities decreased significantly, and this decrease was accompanied by a reduction in the steady-state mRNA levels of AC isoforms V and VI (31).

In conclusion, our results are the first to demonstrate specific patterns of AC isoform expression in primary cultures of various rat EC types. Also, we showed that the AC isoform expression profile in isolated EC significantly differs from that in their parent tissues. Thus, in addition to the previously known tissue specificity of AC isoform expression, our data

suggest unique EC type specificity, which varies from that of the whole organ. Finally, these results support the notion that one of the factors mediating differential regulation of the cAMP cascade in EC in various locations within the vascular tree might be quantitative differences in the levels of AC isoforms expressed in each EC type.

ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid (to P.I.L.) from the American Heart Association (Wisconsin Affiliate), from the National Aeronautics and Space Administration (NASA NAG9-651), and from the Mt. Sinai Research Foundation, as well as by a BDCR grant-in-aid from Marquette University (to B.R.U.).

REFERENCES

1. Barritt, G.J. (1992) *Communication within animal cells,* Oxford Science Publications, New York.
2. Choi, E., Xia, Z., Villacres, E.C., and Storm, D.R. (1993) *Curr. Opin. Cell Biol.* **5**: 269-273.
3. Iyengar, R. (1993) *FASEB J.* 7: 768-775.
4. Wei-Jen, T., Krupinski, J., and Gilman, A.G. (1991) *J. Biol. Chem.* **266**: 8595-8603.
5. Feinstein, P.G., Schrader, K.A., Bakalyar, H.A., Tang, W.J., Krupinski, J., Gilman, A.G., and Reed, R.R. (1991) *Proc. Natl. Acad. Sci. USA* **88**: 10173-10177.
6. Bakalyar, H.A. and Reed, R.R. (1990) *Science* **250**: 1403-1406.
7. Gao, B. and Gilman, A.G. (1991) *Proc. Natl. Acad. Sci. USA* **88**: 10178-10182.
8. Ishikawa, Y., Katsushika, S., Chen, S., Halnon, N.J., Kawabe, J.I., and Homcy, C.J. (1992) *J. Biol. Chem.* **267**: 13553-13557.
9. Krupinski, J., Lehman, T.C., Frankenfeld, C.D., Zwaagstra, J.C., and Watson, P.A. (1992) *J. Biol. Chem.* **267**: 24858-24862.
10. Cali, J.J., Zwaagstra, J.C., Mons, N., Cooper, D.M.F., and Krupinski, J. (1994) *J. Biol. Chem.* **269**: 12190-12195.
11. Xia, Z., Choi, E., Wang, F., Blazynski, C., and Storm, D.R. (1993) *J. Neurochem.* **60**: 305-311.
12. Xia, Z., Choi, E., Wang, F., and Storm, D.R. (1992) *Neurosci. Lett.* **144**: 169-173.
13. Pieroni, J.P., Miller, D., Premont, R.T., and Iyengar, R. (1993) *Nature* **262**: 679.
14. Katsushika, S., Chen, L., Kawabe, J.I., Nilakantan, R., Halnon, N.J., Homcy, C.J., and Ishikawa, Y. (1992) *Proc. Natl. Acad. Sci. USA* **89**: 8774-8778.
15. Premont, R.T., Chen, J., Ma, H.W., Ponnapalli, M., and Iyengar, R. (1992) *Proc. Natl. Acad. Sci. USA* **89**: 9809-9813.
16. Zetter, B.R. (1988) In *Endothelial cells,* Vol. II (ed. U.S. Ryan) CRC Press, Boca Raton, FL, pp. 63-79.
17. Gerritsen, M.E. (1987) *Biochem. Pharmacol.* **36**: 2701-2711.
18. Gumkowski, F., Kaminska, G., Kaminski, M., Morrissey, L.W., and Auerbach, R. (1987) *Blood Vessels* **24**: 11-23.
19. Manolopoulos, V.G. and Lelkes, P.I. (1993) *Biochem. Biophys. Res. Commun.* **191**: 1379-1385.
20. Manolopoulos, V.G., Samet, M.M., and Lelkes, P.I. (1995) *J. Cell. Biochem.* in press.

21. Kern, P.A., Knedler, A., and Eckel, R.H. (1983) *J. Clin. Invest.* **71**: 1822-1829.
22. Lum, H., Andersen, T.T., Siflinger-Birnboim, A., Tiruppathi, C., Goligorsky, M.S., Fenton, J.W., and Malik, A.B. (1993) *J. Cell Biol.* **120**: 1491-1499.
23. McGuire, P.G. and Orkin R.W. (1987) *BioTechniques* **5**: 546-554.
24. Papadimitriou, E., Unsworth, B.R., Maragoudakis, M.E., and Lelkes, P.I. (1993) *Endothelium* **1**: 207-219.
25. Lelkes, P.I., Ramos, E.M., Chick, D.M., Liu, J., and Unsworth, B.R. (1994) *FASEB J.* **8**: 1177-1182.
26. Tso, J.Y., Sun, X-H., Kao, T-H., Reece, K.S., and Wu, R. (1985) *Nucleic Acid Res.* **13**: 2485-2502.
27. Rimarachin J.A., Szabo P., Norcross, J., and Weksler, B.B. (1992) *In Vitro Cell. Dev. Biol.* **28A**: 705-707.
28. Youdim M.B.H., Banerjee, D.K., and Pollard H.B. (1984) *Science* **224**: 619-621.
29. Kawabe, J., Iwami, G., Ebina, T., Ohno, S., Katada, T., Ueda, Y., Homcy, C.J., and Ishikawa, Y. (1994) *J. Biol. Chem.* **269**: 16554-16558.
30. Tobise, K., Ishikawa, Y., Holmer, S.R., Im, M., Newell, J.B., Yoshie, H., Fujita, M., Susannie, E.E., and Homcy, C.J. (1994) *Circ. Res.* **74**: 596-603.
31. Ishikawa, Y., Sorota, S., Kiuchi, K., Shannon, R.P., Komamura, K., Katsushika, S., Vatner, D.E., Vatner, S.F., and Homcy, C.J. (1994) *J. Clin. Invest.* **93**: 2224-2229.