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THE EFFECT OF SALMON CALCITONIN ON BLOOD-IONIZED CALCIUM IN THE PRESENCE OF ANTI-SALMON CALCITONIN ANTIBODIES (FROM PAGETIC PATIENTS) IN YOUNG RABBITS

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ABSTRACT

The occurrence of specific anti-SCT antibodies (Ab) in 65% of pagetic patients treated by nasal salmon calcitonin (SCT) immunologically confirms the bioavailability of SCT when given by the nasal route. The affinity constant of these Ab varies from 1.12 to 4.47 10^9 L/mol and their titer from 0.4 to 21 10^{-9} mol/L. Injection of 1 IU/kg SCT to young rabbits produced a significant decrease ($P < 0.05$) between 60 and 180 minutes in ionized blood calcium (Ca^{2+}) measured by a highly accurate method—the ion-selective electron method. Addition of an equimolar amount of purified specific Ab to SCT did not decrease the intensity of the hypocalcemic response to SCT but did appear to induce an extension of this effect since Ca^{2+} was still significantly decreased ($P < 0.05$) after 210 minutes. There is no evidence from these data that specific anti-SCT Ab, even with high binding affinity, were able to neutralize SCT activity, Ab binding to SCT could result in an extension of SCT activity, possibly by a reduction in the rate of SCT catabolism, but this mechanism remains to be elucidated.

INTRODUCTION

Calcitonin (CT) was first administered to humans in 1966,[1] and many studies[2–4] have shown beneficial effects of porcine calcitonin, salmon calcitonin (SCT), and human calcitonin on the clinical and biological disturbances of diseases characterized by excessive bone remodeling. The amino acid sequence of porcine calcitonin and SCT differ considerably from the human hormone,[5] and specific antibodies (Ab) develop in a significant proportion of treated patients.[6,7] Controversy remains on the functional importance of these Ab.[6–9] In Paget's disease of bone, resistance to CT developing during treatment was ascribed to Ab by some authors,[7,8] while others[9] found that the presence of significant Ab titers had no relationship

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to the development of such resistance; the latter opinion was supported by the fact that resistance to CT also occurs in patients treated with human calcitonin, which is not antigenic.[10] The recent development of a new active mode of administration of SCT,[11,12] nasal spray, without the classic side effects encountered during parenteral CT,[13] allows SCT to be considered a potential preventive therapy for postmenopausal osteoporosis. Since SCT appears to be promising for this indication,[14] long-term or even lifelong treatment may be necessary. It is thus of real importance to assess whether anti-SCT Ab could inactivate salmon calcitonin.

The best way to investigate whether Ab exert a functionally significant blocking action on exogenous CT is to measure their influence on the acute hypocalcemic response to CT.[6] In pagetic patients, the degree of hypocalcemia is dependent on the prevailing rate of bone turnover.[15] The assessment of functional significance of Ab developed during treatment must therefore consider this variable.

Since one of the authors[16] developed a highly resolutive method to measure changes in ionized calcium concentration in the blood of young rabbits, we used this technique in the present study to investigate the effect of SCT incubated with purified endogenous antibodies obtained from pagetic patients treated by nasal SCT to measure the relationship between the presence of anti-SCT Ab and the effectiveness of the hormone.

MATERIALS AND METHODS

Patients

Eleven patients (six men, five women), aged 58 to 88 years (mean ± SD, 67 ± 10 years) with clinical, radiologic, and biologic features of Paget's disease of bone were treated for six months with 200 IU/day (n = 5) or 400 IU/day (n = 6) of nasal SCT and thereafter investigated for the presence of anti-SCT antibodies.

Detection and Characterization of Anti-SCT Antibodies

Salmon calcitonin was labeled with ^{125}I using the chloramine T method[17] and was purified by filtration on Sephadex G15 and G50. This tracer was incubated with several dilutions of serum from pagetic patients in 0.05 mol of phosphate buffer for 96 hours at 4 °C. After incubation, free and bound labeled SCT were separated by adsorption of free SCT on dextran/charcoal (0.5%/5%),[18] whereas SCT bound to Ab remained in solution. A control procedure was performed using serum of normal subjects who had never received SCT.

Ab were considered to be present when radioactivity remaining in the

surpernatant, unadsorbed to dextran-charcoal, corresponded to at least twice the values reported in controls and exceeded 10% of the total radioactivity.

To characterize the nature of endogenous anti-SCT Ab, an enzyme-linked immunosorbent assay (ELISA)[19] was performed. SCT (10 μg/L) was physically coated onto polystyrene tubes. The serum of pagetic patients was then incubated at several dilutions (0.1 ml of serum diluted from 1/1 to 1/16) for 48 hours at 20 °C. The tubes were then washed and rinsed with 0.05 mol of phosphate buffer and specific anti-IgG, IgM, IgA conjugated to peroxidase were added. Incubation continued for two hours at 20 °C. After again washing and rinsing, the addition of a chromogene followed by illumination at 405 nm revealed peroxidase linkage to the wall of the tubes and the nature of the Ab.

Purification of Gammaglobulins

Immunoglobulins were prepared from a volume of 50 ml of serum. Immunoglobulins were first precipitated using the sodium sulfide procedure described by Kekwick.[20] Gammaglobulins (IgG) were separated by chromatography on a Protein A-Sepharose CL–4B[21] (Pharmacia, LKB, Brussels, Belgium)[21] and lyophilizated.

Scatchard Analysis

Binding of ^{125}I-SCT to endogenous Ab was inhibited by adding increasing amounts of unlabeled SCT to the incubation medium. A global volume of 0.4 ml, including 0.1 ml of labeled SCT, 0.1 ml of 0.05-mol phosphate buffer, 0.1 ml of serum (diluted from 1/4 to 1/16 following the amount of IgG previously determined), and 0.1 ml of unlabeled SCT, was incubated for 96 hours at 4 °C. Separation of free and bound ^{125}I-SCT was performed by adsorption of free SCT to dextran-charcoal.[18]

From these inhibition curves, the affinity and concentration of Ab were calculated according to Scatchard analysis.[22] This procedure was reported for serum from pagetic patients and also for purified IgG. All results were expressed considering 1 IU of SCT = 2×10^{-7} g = 5.55×10^{-11} mol.[23]

Hypocalcemia Test

We compared the hypocalcemic effect of the following intravenous (IV) injections into young rabbits: (1) SCT in acetic acid-sodium acetate buffer (pH 6.5) 82.03 mol, 1 IU/kg (two assays); (2) SCT in the same acetic acid-sodium acetate buffer 82.03 mol (pH 6.5; 1 IU/kg) with an equimolar amount of extracted anti-SCT Ab from pagetic patients, with the experi-

ment repeated using the four Ab with the highest affinities (4.47×10^9 L/mol, 4.39×10^9 L/mol, 3.59×10^9 L/mol, and 2.76×10^9 L/mol); (3) anti-SCT Ab from pagetic patients in the same acetic acid-sodium acetate buffer 82.03 mol (pH 6.5), without SCT and with the same amounts of Ab as were injected in 2; (4) SCT in the same acetic acid-sodium acetate buffer 82.03 mol (pH 6.5; 1 IU/kg) with an equimolar amount of nonspecific human gamma-G-immunoglobulins; and (5) nonspecific human gamma-G-globulins at the same incubation as in 4, diluted in the same acetate buffer pH 6.5.

Ionized Calcium Determinations

The ionized calcium (Ca^{2+}) ion-selective electron method (Ca^{2+} IESM)[16] involved continuous measurement of the ionized calcium concentration in the blood of young rabbits. The blood was transported by a peristaltic pump at a flow rate of 18 ml/hr from the femoral artery via indwelling catheters to the femoral vein through a system containing Ca^{2+}-selective liquid membrane electrodes based on the neutral carrier ETH 1001. A second peristaltic pump transported a reference electrolyte solution at a rate of 0.9 ml/hr past a reference electrode, where it met and mingled with the circulating blood. The mixture of blood and reference solution was then returned to the circulating system of the animals. The concentration of ionized calcium in the blood was determined potentiometrically. Differences in potential between the reference electrode and the Ca^{2+}-selective electrodes were measured relative to a common electrode. The differences were amplified and the analog signal produced by the amplifier was processed by a digital voltmeter and recorded continuously as a function of time, the difference in voltage being proportional to the Ca^{2+} concentration. The concentration of ionized Ca^{2+} in the blood (1.1 to 1.2 mmol/L) is about 10^{-5} higher than the detection limit of the electrodes in aqueous solution of calcium (10^{-5} to 10^{-6} mmol/L). The method can detect a change of 0.003 mmol/L (0.3%) in Ca^{2+} concentration. It can also detect the effect of as little as 0.1 IU/kg of SCT on blood levels, with an interindividual variation of some 10% to 20%. The change in blood-ionized calcium was expressed in percentage of pretest value regarded as a baseline.

Statistical Analysis

A paired Student's *t* test was used for assessing the level of significance of the fluctuation of the Ca^{2+} reported at different times compared with that at the beginnning of the test. To compare the responses to the different infusions, we developed a Zerbe's randomization analysis method.[24]

RESULTS

After six months of treatment with nasal SCT, specific anti-SCT Ab were present in seven of 11 treated pagetic patients, four of whom were treated with a daily dose of 400 IU/day and three receiving 200 IU/day. All anti-SCT Ab belonged to the IgG class. The constant of affinity (Ka) of the Ab varied from 1.12 to 4.47 $\times 10^9$ L/mol (mean $\pm$ SD, 2.79 $\pm$ 1.17 $\times 10^9$ L/mol) and their titer from 0.4 to 21 $\times 10^{-9}$ mol (mean $\pm$ SD, 8.2 $\pm$ 6.7 $\times 10^{-9}$ mol). The Ka of anti-SCT Ab were identical, whether determined directly from serum or from purified IgG.

IV injection of 1 IU/kg SCT to the young rabbits led to a significant ($P < 0.05$) decrease in Ca^{2+} between 60 and 180 minutes and to a maximum peak decrease obtained after 120 minutes ($-27 \pm 0.5\%$) (Table I). IV injection of 1 IU/kg SCT with an equimolar amount of anti-SCT Ab was followed by a significant decrease ($P < 0.05$) of Ca^{2+} between 30 and 210 minutes, while the maximum decrease was reached after 120 minutes ($-21.4 \pm 1\%$). No statistical difference appeared between the decrease of Ca^{2+} reported after SCT or SCT + Ab injection. However, a significant Ca^{2+} decrease reported after SCT + Ab began earlier (30 versus 60 minutes) and lasted longer (180 versus 210 minutes) than the one induced by SCT alone. After 210 minutes, while the effect of SCT alone had disappeared, Ca^{2+} continued to decrease from 5.2% to 16% after SCT + Ab injection; this decrease was significant (Table I).

Injection of SCT and an equimolar amount of nonspecific human gamma-G-immunoglobulins produced a maximum Ca^{2+} decrease after 150 minutes; this decrease was not statistically different from the decrease in Ca^{2+} reported after injection of SCT alone or SCT and specific anti-SCT gamma-G-globulins. Injection of anti-SCT gamma-G-globulins or nonspecific gamma-G-immunoglobulins without SCT did not induce any statistically significant change in Ca^{2+} (Table II).

DISCUSSION AND CONCLUSIONS

Some reports[11,12,14,25] have described a biological and clinical activity for SCT administered through the nasal mucosa. However, the occurrence of specific anti-SCT Ab in patients who never had experienced SCT by a route other than the nose is reported here for the first time and should be considered as the immunologic proof of the biodispensibility of SCT after nasal administration. Antibodies developed within the first six months of nasal SCT treatment in seven of 11 (63.5%) patients, and this incidence is similar to that found in other series using parenteral administration.[8] All the Ab belonged to the IgG class, confirming data previously obtained from parenteral SCT by other techniques.[7,25] No circulating anti-SCT IgA was found, although we did not perform measurement within the nasal mucosa

Table I. Variations of blood-ionized calcium (Δ%) in the young rabbit after intravenous administration of 1 U/kg of synthetic salmon calcitonin complexed or not complexed with anti-calcitonin antibodies.

				CT (1 U/kg) and Equimolar Amount of					
	CT Alone (1 U/kg)			Specific Anti-SCT Gamma-G-Globulins					Nonspecific
Time (hr)	CT1	CT2	Mean ± SEM	Ab1	Ab2	Ab3	Ab4	Mean ± SEM	Gamma-G-Globulins
0	0	0	0	0	0	0	0	0	0
0.08	0.12	0.26	0.19 ± 0.07	−0.56	−0.28	0.36	−0.20	−0.17 ± 0.19	0.26
0.16	0.02	−1.14	−0.56 ± 0.58	0.94	0.98	−2.34	−1.74	−0.54 ± 0.87	−1.07
0.25	−1.23	−2.69	−1.96 ± 0.73	0.69	1.32	−4.36	−4.64	−1.75 ± 1.60	−2.24
0.5	−8.72	−12.01	−10.37 ± 1.65	−3.17	−5.23	−7.45	−12.25	−7.03 ± 1.95	−8.07
1	−20.72	−22.33	−21.53 ± 0.81	−7.67	−14.06	−15.06	−19.16	−13.99 ± 2.38	−13.65
1.5	−25.47	−26.66	−26.06 ± 0.60	−16.01	−18.51	−16.40	−23.58	−18.63 ± 1.74	−18.45
2	−26.61	−27.56	−27.09 ± 0.48	−20.89	−21.06	−19.34	−24.44	−21.43 ± 1.07	−20.94
2.5	−25.41	−25.45	−25.43 ± 0.02	−19.13	−21.13	−17.75	−20.83	−19.71 ± 0.79	−21.47
3	−17.79	−16.73	−17.26 ± 0.53	−17.89	−16.46	−16.25	−15.31	−16.48 ± 0.53	−20.61
3.5	−3.35	−1.87	−2.61 ± 0.74	−16.42	−5.60	−11.95	−7.20	−10.29 ± 2.45	−14.75
4	−0.14	−0.46	−0.30 ± 0.16	−13.36	0.46	−5.82	−1.71	−5.11 ± 3.04	−9.62
4.5	−0.05	0.91	0.43 ± 0.48	−10.29	1.42	0.75	−1.40	−2.38 ± 2.70	−0.50
5	0.29	0.84	0.57 ± 0.28	−8.07	0.24	4.45	−1.38	−1.19 ± 2.60	−0.55
0–5 hr (Δ% · hr)	−63.16	−64.89	−64.03 ± 0.50	−63.89	−49.09	−53.48	−62.68	−57.29 ± 3.59	−63.60

Table II. Variations of blood-ionized calcium (Δ%) in the young rabbit after intravenous administration of 1 U/kg of synthetic salmon calcitonin complexed or not complexed with anti-calcitonin antibodies. Data given are means ± SEM.

Time (hr)	CT Alone (1 U/kg)	CT (1 U/kg) and Equimolar Amount of Specific Anti-SCT Gamma-G-Globulins
0	0	0
0.08	0.19 ± 0.07	−0.17 ± 0.19
0.16	−0.56 ± 0.58	−0.54 ± 0.87
0.25	−1.96 ± 0.73	−1.75 ± 1.60
0.5	−10.37 ± 1.65	−7.03 ± 1.95
1	−21.53 ± 0.81	−13.99 ± 2.38
1.5	−26.06 ± 0.60	−18.63 ± 1.74
2	−27.09 ± 0.48	−21.43 ± 1.07
2.5	−25.43 ± 0.02	−19.71 ± 0.79
3	−17.26 ± 0.53	−16.48 ± 0.53
3.5	−2.61 ± 0.74	−10.29 ± 2.45
4	−0.30 ± 0.16	−5.11 ± 3.04
4.5	0.43 ± 0.48	−2.38 ± 2.70
5	0.57 ± 0.28	−1.19 ± 2.60
0–5 hr (Δ% · hr)	−64.03 ± 0.50	−57.29 ± 3.59

surfactant. Ab rose at the same rate whether 200 or 400 IU/day of nasal SCT was given, confirming the lack of relationship between their occurrence and the dose administered.[2]

If any blocking activity was devoted to anti-SCT Ab, considering that 1 mol of Ab could counteract 1 mol of SCT and that 1 mg of SCT contains about 5,000 IU,[23] the titer of specific Ab measured in our population would permit the inhibition of SCT amounts up to 378 IU/L. Thus, in prospective long-term SCT treatment using doses in a range of 50 to 100 IU/day and even lower, the ability of Ab to neutralize SCT activity remains a major question to be elucidated.

In a classic test comparing the hypocalcemic effect of SCT in vivo before and after occurrence of Ab, a bias might appear to be due to the relative imprecision of Ca measurement and to changes, within a relatively long period of time, in baseline Ca before SCT injection.[26] Our methodology, using a short-term procedure as well as a highly accurate and precise methodology for determining Ca^{2+}, avoids this bias.[16]

Injection of SCT and an equimolar amount of specific Ab was followed in every test by a fall in Ca^{2+} within a similar range as observed when SCT was injected alone. The hypocalcemic effect of the association SCT + Ab is not related to a bipolar effect of Ab—blocking SCT activity on osteoclasts on the one hand and producing hypocalcemia by an unknown mechanism on the other—since injection of Ab alone was not followed by any change in Ca^{2+}.

Since we selected the Ab presenting the highest Ka from our population of pagetic patients for this investigation, these results are not in

agreement with the hypothesis that Ab of very high-binding affinity would be involved to produce resistance against SCT.[6]

Because the young rabbit may be considered to have had no change in bone turnover during the period of investigation, valid comparisons between the hypocalcemic responses may be made. No significant difference appeared during the entire test between the hypocalcemic effect of SCT and SCT + Ab. In agreement with Hosking and coworkers,[6] we used the area under the Ca^{2+} versus time curve according to Zerbe's analysis[24] to compare the responses with or without Ab, rather than the maximum decrease in Ca^{2+}, because the former incorporates an expression of the temporal relationship of this change. The analysis of the Ca^{2+} versus time curves showed no statistical difference, regardless of whether Ab were present.

Therefore, we do not support the hypothesis that anti-SCT Ab, even with a high affinity, is responsible for the resistance occurring during SCT treatment through a competition between SCT and its receptor on the osteoclast.[8]

There may be paradoxical increases in serum calcium immediately after calcitonin administration[27]; considering the small size of our sample, no hypothesis may be drawn from the delay in the occurrence of Ca^{2+} decrease with SCT alone, but the explanation for the remaining hypocalcemic effect of SCT and antigenous-specific antibodies is not clear. It may be that SCT is bound to antibodies with high affinity in such a manner as to not interfere with hormone receptor interaction and that this nonbiologically blocking binding to the Ab might delay the catabolism of SCT and prolonged biological half-life of SCT. Ongoing studies will determine the impact of Ab occurrence on the half-life of injected SCT.

The occurrence of specific Ab in patients treated with nasal SCT confirms the bioavailability of the compound when administered by the nasal route. No evidence appears that anti-SCT Ab is able to block SCT activity even if Ab possesses a high binding affinity. Similar results[28,29] were shown for diabetic patients treated with insulin who developed anti-insulin Ab, which does not usually interfere with the therapeutic effect of insulin. Furthermore, binding of Ab to SCT appears to result in an extended hypocalcemic effect of SCT for which the mechanism remains to be elucidated.

References:

1. Foster GV, Joplin GF, MacIntyre I, et al. Effect of thyrocalcitonin in man. *Lancet* 1966; 1:107.
2. Singer FR. Human calcitonin treatment of Paget's disease of bone. *Clin Orthop Rel Res* 1977; 127:86–93.
3. Shai F, Baker R, Wallach S. The clinical and metabolic effects of porcine calcitonin on Paget's disease of bone. *J Clin Invest* 1971; 50:1927–1940.

4. Haddad JG, Birge SJ, Avioli LV. Effects of prolonged thyrocalcitonin administration on Paget's disease of bone. *New Engl J Med* 1970; 283:549–555.

5. Niall HD, Keutmann HT, Copp DH, Potts JT. Amino acid sequence of salmon ultimobranchial calcitonin. *Proc Natl Acad Sci USA* 1969; 64:771–778.

6. Hosking DJ, Denton LB, Cadge B, Martin TJ. Functional significance of antibody formation after long-term salmon calcitonin therapy. *Clin Endocrinol* 1979; 10:243–252.

7. Singer FR, Aldred JC, Neer RM, et al. An evaluation of antibodies and clinical resistance to salmon calcitonin. *J Clin Invest* 1972; 51:2331–2338.

8. Singer FR, Fredericks RS, Minkin C. Salmon calcitonin therapy for Paget's disease of bone. The problem of acquired resistance. *Arthritis Rheum* 1980; 23:1148–1153.

9. Derose J, Singer FR, Avramides A, et al. Response of Paget's disease to porcine and salmon calcitonins. *Am J Med* 1974; 56:858–866.

10. Greenberg PB, Doyle FM, Fisher MT, et al. Treatment of Paget's disease of bone with synthetic human calcitonin. *Am J Med* 1974; 56:867–870.

11. Reginster JY, Albert A, Franchimont P. Effect of nasal administration of synthetic salmon calcitonin in Paget's bone disease. *Calcif Tissue Int* 1985; 37:577–580.

12. Reginster JY, Denis D, Albert A, Franchimont P. Assessment of the biological effectiveness of nasal synthetic salmon calcitonin (SSCT) by comparison with intramuscular (i.m.) or placebo injection in normal subjects. *Bone Mineral* 1987; 2:133–140.

13. Reginster JY, Franchimont P. Side effects of synthetic salmon calcitonin given by intranasal spray compared with intramuscular injection. *Clin Exp Rheum* 1985; 3:155–157.

14. Reginster JY, Denis D, Albert A, et al. 1-year controlled randomization trial of prevention of early postmenopausal bone loss by intranasal calcitonin. *Lancet* 1987; 2:1481–1483.

15. Bijvoet OLM, Vandersluysveer J, Jansen AP. Effects of calcitonin on patients with Paget's disease, thyrotoxiosis and hypercalcaemia. *Lancet* 1968; 1:876–881.

16. Azria M, Attinger M, Schupfer H, Tanner P. Comparison of the hypocalcaemic activities of three calcitonins using a new biological method. In: Doepfner W, ed. *Calcitonin 1984*. Excerpta Medica Publ.; Amsterdam, Hong-Kong, Princeton, Sydney, Tokyo 1984; 104–110.

17. Greenwood FC, Hunter WM, Glover JS. Preparation of iodine-131 labelled human growth hormone of high specific radioactivity. *Biochem J* 1963; 89:114–123.

18. Franchimont P, Heynen G. *Parathormone and calcitonin radioimmunoassay in various medical and osteoarticular disorders*. Masson Eds. New-York, Paris, Barcelone, Milan, 1976; 134 p.

19. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay, ELISA. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. *J Immunol* 1972; 109:129–135.

20. Kekwick RA. The serum proteins in multiple myelomatosis. *Biochem J* 1940; 34:1248–1257.

21. Forsgren A, Sjoquist J. Protein A from *S. aureus*. I. Pseudoimmune reaction with human gamma-globulin. *J Immunol* 1966; 97:822–827.

22. Scatchard G. The attractions of proteins for small molecules and ions *Ann NY Sci* 1949; 51:660–672.

23. Guttmann S. Chemistry and structure-activity relationship of natural and synthetic calcitonins. In: *Calcitonin 1980, chemistry, physiology, pharmacology and clinical aspects.* Pecile A, eds.; Excerpta Medica Amsterdam, Oxford, Princeton Publ. 1981; 11–24.

24. Zerbe GO. Randomization analysis of the completely randomized design extended to growth and response curves. *J Am Stat Assoc* 1979; 74:215–221.

25. Singer FR, Bloch KL. Antibodies and clinical resistance to salmon calcitonin. *Clin Res* 1972; 20:220.

26. Riggs BL, Arnaud CD, Goldsmith RS, et al. Plasma kinetics and acute effects of pharmacologic doses of porcine calcitonin in man. *J Clin Endocrinol* 1971; 33:115–127.

27. Mohamadi M, Becker KL, Bivins LE. Paradoxical effect of salmon calcitonin on serum calcium: Studies in intact and thyroparathyroidectomized men and dogs. *Acta Endocrinol* 1975; 79:700–708.

28. Berson SA, Yalow RS. Quantitative aspects of the reaction between insulin and between insulin binding antibody. *J Clin Invest* 1959; 38:1996.

29. Haddad JG, Caldwell JG. Calcitonin resistance: Clinical and immunological studies in Paget's disease of bone treated with porcine and salmon calcitonin. *J Clin Invest* 1972; 51:3133–3141.