The mechanism of cyclosporine-A induced antiinflammation

V.Nimet iZGUT, Giilsen ONER

Dept. of Physiology, Medical School of Akdeniz University, ANTALYA, TURKEY

In order to investigate the mechanism by which cyclosporine-A produces antiinflammatory action, we carried out an in vitro study using peritoneal cells of male albino rats weighing 130-170 g.

Isolated rat peritoneal cells were divided into 3 groups and incubated at 37°C under 5% CO_2 and 95% O_2 in 1 ml of RPMI-1640 for 16 hours. The cells in group one were kept unstimulated while 10 M of dexamethasone and 10 ug *of cyclosporine-A were used for the stimulation of the cells in group 2 and 3 respectively. Fifteen minutes before terminating the incubation all tubes were added 2 \xM of A23187, a calcium ionophore. Arachidonic acid levels were measured using HPLC in the supernatant of cells from all groups as well as the amount of proteins synthesized during 16 hours were measured by Lowry. In control tubes the amount of arachidonic acid released from the membrane of 10X10^6 cells was 21.34±3.48 pg and the total synthesized protein was 1178.81±190.45* ug *during 16 hrs. With the treatment of 10^{-} M of dexamethasone, the cells produced 1658.92±234.64 mg of protein and released 12.79±6.49 pg of arachidonic acid. In the cells stimulated with 10 mg of Cyclosporine-A, protein synthesis increased to 1385.05±202.60* pg, *release of arachidonic acid decreased to 17.68±2.38pg during incubation period. Despite general rise in proteins seen in all fractions of dexamethasone stimulated cells, cyclosporine-A caused only an increase in the proteins of fractions 6 and 7.*

As an index of antiinflammatory action, the antiphospholipase A_2 activity of proteins in all fractions was measured and only in fraction 11 of dexamethasone treated group and in fraction 6 of cyclosporine-A stimulated cells showed lipocortin like activity. [Turk J Med Res 1993 11(5): 213-216]

Key Words: Anti-PLA2, Cyclosporine-A, Glucocorticoids, Lipocortin

Recently the suppression of immune system has been aimed for the prevention of allograft rejection and treatment of autoimmune diseases (1-3).

Among many potent immunodepressive drugs, cyclosporin-A (Cyc-A) is widely used for both its anti-inflammatory and immunosupressive effects (2,4-6). Despite the presence of numerous reports about the action mechanism of antiinflammatory effects of steroid which are the most common drugs used for antiinflammation, the mechanisms by which Cyc-A induces antiinflammation is obscure. The steroid induced anti-inflammation is attributed to the synthesis of a cytosolic protein called lipocortin which inhibits the activity of phospholypase A_2 enzyme (7-10). In inflammatory processes, lipid mediators such as prostaglandins (PGs) and leucotrienes (LTs) are produced by mediation of phospholypase A_2 enzyme from the membrane derived arachidonic acids (AA) (11). Although the inhibition of the production of inflammatory lipid mediators (12-14) during the Cyc-A treatment strongly suggest the similarity between the action mechanism of antiinflammatory effects of steroids and Cyc-A (6,15), there is no definite report explaining the reason of this reduction in the release of AA products due to Cyc-A treatment.

Because of wide therapeutic usage of Cyc-A, it has been thought that knowing the mechanism of anti-inflammatory effects of this drug may have clinical importance.

This in vitro study has been carried out in order to investigate the mechanism by which Cyc-A produces antiinflammation.

MATERIALS AND METHODS

In this study, the peritoneal cells obtained from male albino rats weighing 130-170 g were used.

1. To determine the effect of Cyc-A on AA production; the peritoneal cells were isolated as described by Blackwell et al (8). 10x10^6 cells in 1ml of RPMI 1640 with 10% fetal calf serum containing tubes were divided into three groups and incubated at 37°C under 95% O_2 and 5% CO_2 for 16 hours. The tubes in group one were kept as control while 10~^{6} M of dexamethasone and 10ug of Cyc-A were added into the cells in group two and three respectively. Two uM of A23187 (a calcium ionophore) was added into the all tubes 15 min before the termination of the incubation. At the end of incubation period all cells were centrifuged at 2000 rpm for 5 minutes at 4°C. The lipids in the supernatants were extracted with the mixture of chloroform: Methanol (3:1 v/v) and their AA

Received: Feb.11, 1992 Accepted: Oct. 17, 1992

Correspondence: V.Nimet iZGUT
Dept. of Physiology, Medical School of
Akdeniz University, Antalya, TURKEY

content was determined by the method of Cockrell at al (16) using High Performance Liquid Chronatography (HPLC) (varian 5020 model) equipped with SPC-18 reverse phase column (150x4 mm).

2. To measure the antiinflammatory action mechanism of Cyc-A, above mentioned experiment was repeated using 2x10^6 cells containing tubes. To prevent the destructive effects of proteases on newly synthesized proteins, all tubes were added 50 uM of phenylmethylsulphonyfluoride, a protease inhibitor during 16 hrs incubation.

Following the incubation period, the cells were disrupted by a homogenizer (TRI-R STIR-R) at 10000 rpm for 5 minutes at 4°C and the homogenates were centrifuged at 4°C for 30 min at 8000 rpm and their supernatants were loaded into a sephadex-G 50-80 column (60x1 cm) equilibrated with ammonium bicorbonate buffer solution at pH:8.0 and 10 min. fractions were collected for 250 min.

The amount of total protein in supernatant as well as in the fractions were measured by the method of Lowry (17).

The molecular weight of proteins present in each fractions were determined by means of the calibration curve prepared with the same sephadex column.

3. To determine the potence of antiphospholypase A_2 activity of the proteins present in the eluents, 50 pg of protein from each fraction was added into the tubes which contain the lipid extract of red blood cell membrane containing 200 mg of protein in 1ml of 100 mM Tris-HCI with 10 mM $CaCl_2$.

After 30 min incubation at room temperature, 100 ng of phospholypase A2 (Sigma# P-6534) was added into all tubes and reaction was terminated after 2 minutes by adding 50 pi of 1 N HCI.

The amount of AA released into the medium was measured using HPLC and the inhibitory effects of eluted proteins on AA release was compared with the control tubes containing only membrane phospholipids and phospholypase A_2 enzyme.

The results were expressed as percent inhibition of total AA produced in control tubes.

Student's "t" tests was used for the statistical evaluation of the results.

RESULTS

The Effect of Cyclosporin-A on Arachidonic Acid Production: In control conditions the amount of AA released from 10x10^5 peritoneal cells was 21.34+3.48 pg/ml. The addition of 10"^{6} M of dexamethason into the medium decreased significantly the amount of AA to 12.79±6.49 pg/ml ($p<0.01$). Cyc-A addition showed significant depressive effect on AA release from the membrane lipids and the mean level of AA was 17.68±2.38 pg/ml in tubes containing 10pg of Cyc-A ($p<0.05$, Figure 1).

The Effect of Cyclosporin A on Protein Synthesis by Peritoneal Cells: In control tubes containing 2x10^6 peritoneal cells 1178.81 ±190.45 pg/ml protein were synthesized during 16 hrs incubation period. Addition of 10"^{6} M of dexamethasone and 10 pg of Cyc-A increased significantly the amount of newly synthesized protein. The mean protein value was 1658.92±234.64 mg/ml ($p<0.001$) and 1385.05±202.60 mg/ml ($p<0.05$), respectively (Figure 2).

As seen in Figure 3 the elution of newly synthesized proteins by 2x10^6 cells through Sephadex G 50-80 column showed that the proteins in control tubes occured mainly between fraction 8 and 11 and the range of their molecular weight was 37500 and 55000 daltons.

Dexamethasone increased the synthesis of protein in all fractions (Figure 4). However Cyc-A caused an elevation in proteins eluted only in fraction 6 and 7 which are absent in both control and dexamethason containing tubes.

The range of molecular weight of the proteins unique for Cyc-A was 55000-67500 dalton (Figure 5).

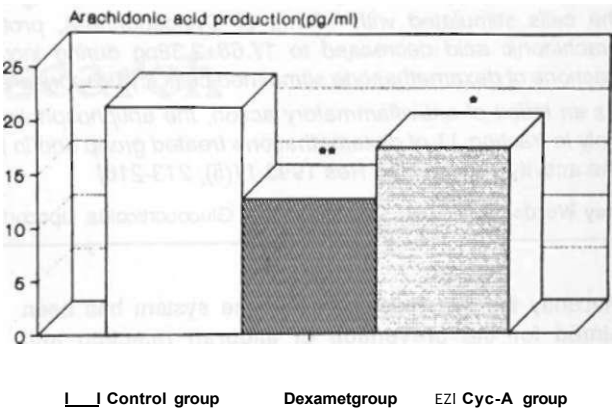


Figure 1. The effect of cyclosporin-A and dexamethasone on arachidonic acid production, *p<0.05, "p<0.01

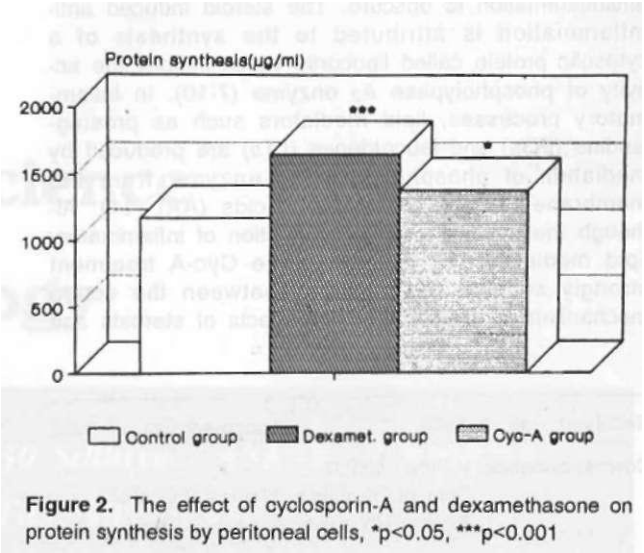
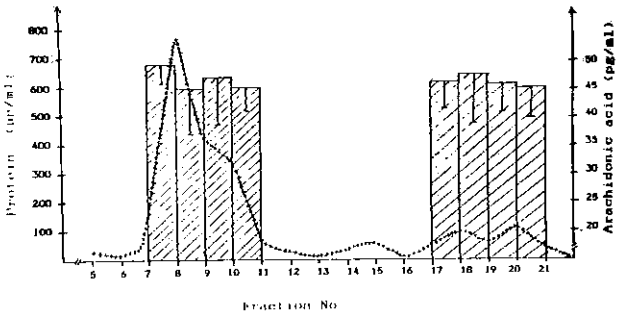


Figure 2. The effect of cyclosporin-A and dexamethasone on protein synthesis by peritoneal cells, *p<0.05, ***p<0.001



Figures. Left scale: The protein profile in the extract of control cells loaded on the sephadex G50-80 column equilibrated with ammonium bicarbonate (pH:8.0) under the flow rate of 0.3 ml/min, right scale: The effect of fractioned protein on the release of arachidonic acid, (***);Protein; ^); Arachidonic acid.

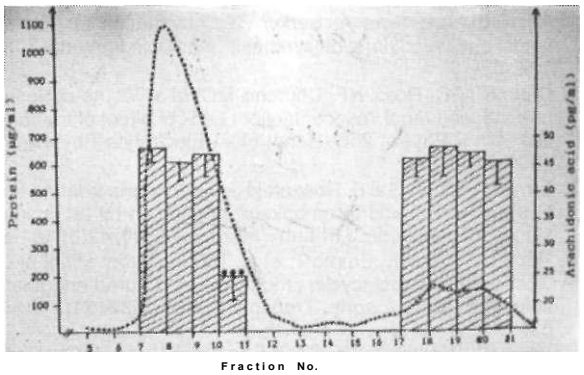


Figure 4. Left scale: The protein profile in dexamethasone treated cells extract loaded on the sephadex G50-80 column equilibrated with ammonium bicarbonate (pH:8.0) under the flow rate of 0.3 ml/min, right scale: The effect of Tractioned protein on the release of arachidonic add, (***); protein, *Y>\:* Arachidonic acid, ***p<0.001.

The Estimation of Phospholipase A2 Activity in the Fractions: None of the protein in the fractions of control tubes showed antiphospholypase A2 activity. AA release in tubes containing protein obtained from control tubes was not significantly different from the value of tubes containing only phospholypase A2 activity (Figure 3).

The protein of 11th fraction of dexamethason containing tubes showed significant depressive effect on phosphoypase A2 activity (Figure 4), and molecular weight of this protein with antiphospholypase A2 activity was 39000 dalton (38000-40000 dalton).

The amount of Cyc-A induced proteins which occur at the 6th fraction was 127.89±45.81pg and 50pg of this protein showed a 8.12±3.86% of^inhibition in AA release. Whereas the protein of fraction 7 did not influence on the amount of release of arachidonic acid (Figure 5).

DISCUSSION

In this experimental study, using isolated rat peritoneal cells it has been observed that Cyc-A inhibits the release of AA from the membrane phospholipids. Despite the presence of this result in the literature (13,14,18,19) its mechanism is unclear. Our present findings show that the antiinflammatory action of Cyc-A can be explained by the presence of a cytosolic protein with a weak antiphospholypase A2 activity.

There are voluminious information in the literature about steroid dependent lipocortin, a protein with antiphospholypase A2 activity (7,8,9,10,20). However there is lack of information about Cyc-A induced protein which shows depressive effect on phospholypase A2 mediated AA release. Since the steroids are widely used in clinics because of their antiinflammatory effects (7,21) the mechanism of steroid dependent antiinflammation has been studied in details (9,10,20,22) and the properties of lipocortin and its molecular weight were defined by Hirata and Iwata (14) and Blackwell et all (8).

Our results corcerning the antihospholypase A2 activity of dexamethasone are well accord with the findings of Blackwell et all (8) who reported that the molecular weight of lipocortin is about 40 Kd. The ineffectiveness of Cyc-A on the protein of fraction 11 which is the fraction of proteins with 40 Kd of molecular weight indicates that antiinflammatory action of Cyc-A can not be attributed to the stimulation of lipocortin. This may be the first observation showing the stimulatory effect of Cyc-A on protein synthesis. Our results clearly showed that antiinflammatory action

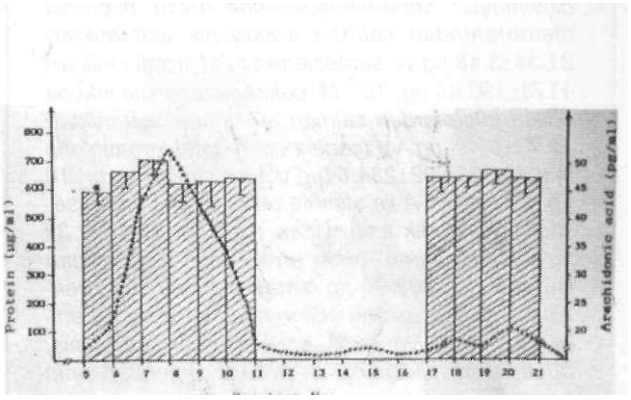


Figure 5. Left scale: The protein profile in cyclosporin-A treated cells extract loaded on the sephadex G50-80 column equilibrated with ammonium bicarbonate (pH:8.0) under the flow rate of 0.3 ml/min, right scale: The effect of fractioned protein on the release of arachidonic acid, (***); Protein (ug/ml),Eg); Arachidonic acid (pg/ml), *p<0.05).

of Cyc-A depends on a unique protein which is synthe tized by Cyc-A and its molecular weight is higher than that of lipocortin and it is absent both in unstimulated control and dexamethason treated cell cultures.

Our results also demonstrated that this Cyc-A induced lipocortin like protein has weak antiphospholpase A2 activity. Fifty pg of dexamethasone induced lipocortin eluted in fraction 11 caused 46.74±8.4% inhibition in AA release whereas the antiphospholypase A2 potence of Cyc-A induced lipocortin like protein has only 8.12±3.86% inhibitory action on the release of AA from membrane phospholipids.

As a results our findings have shown that,

1. Cyc-A stimulates the synthesis of a protein with a weak antiphospholypase A2 activity in peritoneal cell cultures,

2. This protein which has higher molecular weight than that of lipocortin is responsible for the antiinflammatory effect of Cyc-A.

However further studies are needed to arrive at definite conclusion.

Siklosporin-A'nın antiinflamatuar etki mekanizması

Siklosporine-A'nın antiinflamatuar etki mekanizmasını açıklığa kavuşturmak amacı ile tertiplenen bu in vitro çalışmada, ağırlıkları 130-170 g *arasında olan erkek albino sıçanlardan alınan peritoneal hücreler kullanılmıştır.*

Sıçanlardan alınan peritoneal hücreler 3 gruba ayrılıp, 37°C'de %5 CO2 ve %95 oksijen ortamında 16 saat süre ile inkübe edilmiştir. 1. gruptaki hücreler kontrol olarak ayrılıp, stimülasyon yapılmadığı halde, 2. ve 3. gruptaki peritoneal hücreler sırasıyla, 10⁻⁶ M dekzametazon ve 10 pg *siklosporin-A ile inkübe edilmiştir. İnkübasyon süresinin bitiminden 15 dakika önce 2 \xM A23187 (Ca iyonoforu) ilave edilmiş ve ortama salınan arakidonik asit düzeyi yüksek basınçlı sıvı kromatografisi (HPLC) ile ölçülmüştür. Kontrol koşullarında 10x10 hücrenin membranından salınan arakidonik asit miktarı 21.34±3.48 pg ve sentezlenen totalprotein miktarı 1178±190.45* pg, *10⁻⁶ M dekzametazon ile inkübe edilen hücrelerden salınan arakidonik asit miktarı 12.79±6.49 pg ve sentezlenen total proteinlerin miktarı 1658.92±234.64\ıg olarak saptanmıştır. 10 \xg siklosporin-A ile stimüle edilen hücrelerden salınan aradikonik asit miktarı azalarak 17.68±2.38 pg'a inmiş, sentezlenen proteinlerin total miktarı ise 1385.05±202.60* pg *olarak bulunmuştur. Dekzametazon ile inkübe edilen hücrelerden elde edilen tüm fraksiyonlarda protein artışı gözlenmiş, buna karşın, siklosporin-A'ya bağlı protein artışının sadece 6. ve 7. fraksiyonda olduğu saptanmıştır.*

Antiinflamatuar etkinin göstergesi olarak, tüm fraksiyonlardaki proteinlerin anti-PLAz etkisi ölçüldüğünde, dekzametazon grubunda 11. fraksiyonda, siklosporin-A grubunda ise lipocortin benzeri aktivitenin 6. fraksiyonda olduğu gözlenmiştir.
[Turk J Med Res 1993, 11(5): 213-216]

REFERENCES *

1. Jacobs SB, Pletscher LS, Beale MG. Immunosuppressive effects of low doses of glucocorticoids: Effects on autologous and allogeneic mixed leucocyte reactions. J Immunol 1980; 124(6):2812-17.
2. Simmons RL, Wang SC. New horizons in immunosupression. Transplant Proceed 1991; 23(4):2152-56.
3. Zor U, Her E, Talmon J, et al. A new mechanism of hydrocortisone actions in allergy and inflammation. Ann NY Acad Sei 1988; 524:456-8.
4. Kahan BD. Cyclosporine. N Eng J Med 1989; 321 (25): 1725-38.
5. Stahl RAK, Adler S, Baker PJ, et al. Cyclosporin A inhibits prostaglandin E formation by rat mesangial cells in culture. Kidney Int 1989; 35:1116-67.
6. Wiesenberg-Böttcher I, Wanner K, Pignat W. Antiinflammatory effects of cyclosporine-A (CSA) in carragheenan induced pleurisy in rats. Agent and Action 29(1/2):105-7.
7. Blackwell GJ, Flower RJ, Nijkamp FP, et al. Phospholipase A2 activity of guinea pig isolated perfused: Stimulation and inhibition by antiinflammatory steroids. Br J Pharmacol 1978; 62:79-89.
8. Blackwell GJ, Carnuccio R, Di Rosa M, et al. Glucocorticoids induce the formation and release of antiinflammatory and antiphospholipase proteins into the peritoneal cavity of the rat. Br J Pharmacol 1982; 76:185-94.
9. Flower RJ. Lipocortin and the mechanism glucocorticoids. Br J Pharmacol 1988; 94:987-1015.
10. Goodwin JS, Atluru D, Sierakowski S, et al. Mechanism of action of the glucocorticoids. J Clin Invest 1986; 77:1244-50.
11. Aderem AA, Rosen A, Barker KA. Modulation of prostaglandin and leucotriene biosynthesis. CurOpin Immunol 1988; 1:56-62.
12. Churchill PC, Rossi NF, Churchill MC, et al. Acute cyclosporine-induced renal vasocontriction: Lack of effect of theophylline. Am J Physiol 258 (Renal Fluid Electrolyte Physiol, 27) 1990; F41-F45.
13. Erman A, Chen-Gal B, Rosenfeld J. Cyclosporin treatment alters prostanoid and thromboxane production by rat isolated kidney mitochondria. J Pharm Pharmacol 1990; 42(3): 181-5.
14. Hattori Y, Kasai K, Emoto T, et al. The inhibitory effect of cyclosporine on prostacyclin production by cultured endothelial cells from porcine aorta. Transplant Proc 1989; 21(3):3461-63.
15. Voss BL, Hamilton KK, Scott-Samara EN, et al. Cyclosporine suppression of endothelial prostacylin generation. Transplantation 1988; 45(14):793-776.
16. Cockrell CS, Ellis EF. Simple single-step high performance liquid chromatographyc method for the separation of cyclooxygenase and lipoxygenase enzyme metabolites of arachidonic acid. J Chromatog 1984; 308:316-21.
17. Ann M, Markwell K, Haas SM. Protein determination by lowry method. Methods in Enzymology 1981 ; 72:296-303.
18. Rosenthal RA, Chukwugo NA, Ocasio VH, et al. Cyclosporine inhibits endothelial cell prostacyclin production. J Surg Res 1989; 46:593-6.
19. Sraer J, Bens M, Ardaillou R. Dual effects of cyclosporine A on arachidonate metabolism by peritoneal macrophages (Phospholipase activation and partial thromboxane synthase blockage). Biochem Pharmacol 1989; 38(12):1947-54.
20. Flower RJ, Wood JN, Parente L. Macrocortin and the mechanism of action of the glucocorticoids. Adv Inflam Res 1984; 7:61-70.
21. Calignano A, Carnuccio R, DiRosa M, et al. The antiinflammatory effect of glucocorticoid-induced phospholipase inhibitory proteins. Agents and Actions 1985; 16(1/2):60-2.
22. Hirata F, Iwata M. Role of lipomodulin, a phospholipase inhibitory protein in immunoregulation by thymocytes. J Immunol 1983; 130(4): 1930-36.