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EPINEPHRINE SUPPRESSES SECRETION OF VLDL-ASSOCIATED TRIACYLGLYCEROL AND INCREASES TRIACYLGLYCEROL AND PHOSPHOLIPID CONTENTS IN ISOLATED RAT HEPATOCYTES

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ABSTRACT

The effect of epinephrine on triacylglycerol secretion was investigated in isolated rat hepatocytes. The effect appeared at concentrations of more than 1μM and reached a plateau at 10μM. Epinephrine concentration for half of the maximal bioeffect (EC_{50}) was about 1μM. Epinephrine at a concentration of 10μM suppressed triacylglycerol secretion by 33% and increased its cellular content by approximately 18%. The total triacylglycerol content of the system (sum of the cell and the incubation medium) was constant at all concentrations of epinephrine. Time course experiments for triacylglycerol secretion exhibited relatively similar results on the basis of lipid analysis with or without lipid extractions. On the former basis triacylglycerol secretion versus time followed a linear relationship with a slope of 1.47±0.11 μmole TG/3h/g wet liver. Time course curves of cellular lipids revealed that cellular triacylglycerol and phospholipid contents in the presence of epinephrine were higher than the control at all time points, and the difference was constant during time. Furthermore, in the presence of glucose (20 mM) and oleate (0.25 mM), intracellular triacylglycerol content increased markedly (≥45%) whereas cellular phospholipid content remained constant. It is proposed that epinephrine exerts an inhibitory effect on VLDL secretion probably through blocking in the secretory pathway.

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INTRODUCTION

Catecholamines, especially epinephrine, play an important role in regulating lipid and lipoprotein metabolism,[1] and are elevated in response to acute and chronic stress.[2] The liver secretes triacylgylcerol primarily in the form of very low density lipoprotein (VLDL). This requires coordinated synthesis and secretion of other lipids and protein components of VLDL.[3] Catecholamines inhibit acetyl-CoA carboxylase via enhancing its inactive phosphorylated form and hence are antilipogenic.[4] Inhibition of carboxylase activity leads to reduction of the cytosolic malonyl-CoA level and subsequently to channelling fatty

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acid substrate from esterification to the oxidation pathway.[4] Epinephrine inhibits phosphatidate phosphohydrolase, the key enzyme in glycerolipid biosynthesis. This effect is abolished by oleate in isolated rat hepatocytes.[5] Norepinephrine decreases phosphatidylcholine biosynthesis in hepatocytes which is accompanied with the rate diminution of the CTP: phosphocholine cytidylyltransferase step.[6] Norepinephrine increases 3-hydroxy 3-methyl glutaryl-CoA reductase activity while it inhibits cholesterol secretion from rat hepatocytes.[7]

Synthesis, assembly and secretion of VLDL associated components are subject to hormonal and metabolic regulations and recently have been reviewed.[3,8-13] Secretion of VLDL is not only suppressed by calcium-linked agents such as catecholamines,[14-16] prostaglandins,[17] and calcium antagonists,[18-19] but also acts via the cAMP pathway by agents, i.e. glucagon,[20] cAMP derivatives[21] and cAMP dependent protein kinase.[22] Although secretion of VLDL is regulated by both signal transduction systems (i.e. calcium and cAMP pathways), epinephrine acts mainly through α_1-adrenoceptors in the liver.[23] Dose response of α_1-adrenoceptor agonists on hepatocyte calcium has also been reported that demonstrate negative cooperativity.[24-25] In the present study, the effects of epinephrine on triacylglycerol secretion and cellular lipids have been investigated in isolated rat hepatocytes.

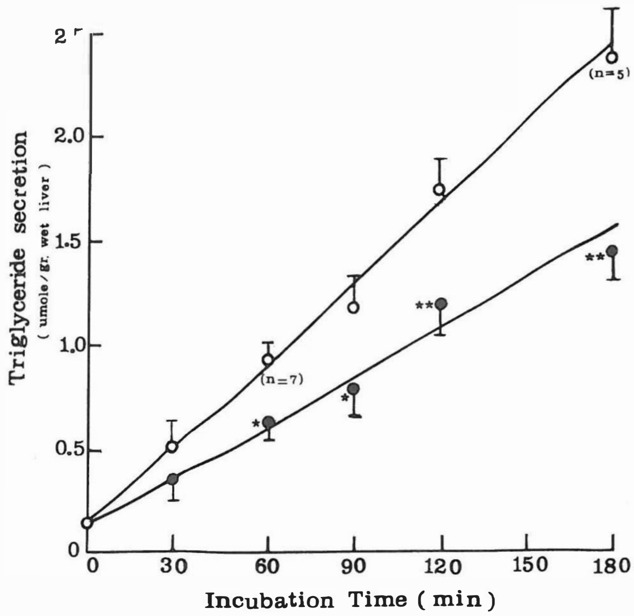

Fig. 1. Time course of triacylglycerol secretion in the absence (○) and presence (●) of epinephrine (10μM) in isolated rat hepatocytes. Hepatocytes at concentrations of 8.0±0.5 mg protein/mL were incubated in KRB containing 20 mM glucose and 0.25 mM oleate in a total volume of 4 mL at 37°C. Triacylglycerol in the incubation medium was measured directly, without lipid extraction. It is assumed that one μmole of triacylglycerol is equal to 885 μg and one gram of wet weight of liver is equal to 158 mg of total cell protein. Results are expressed as mean ± S.E. of four interassays performed at least in three different cell preparations shown in parenthesis. *, ** indicate that the corresponding value is significantly different from its respective control at $p \leq 0.025$ amd $p \leq 0.01$ confidence levels, respectively.

MATERIALS AND METHODS

Chemicals

Epinephrine, oleic acid and bovine serum albumin (essential fatty acid free) were obtained from Sigma (USA). Collagenase (5000 U/mg protein), EDTA, and amino naphthol sulphonic acid were purchased from Merck Chemical Co. Ltd. All other chemicals used were reagent grade.

Hepatocyte isolation

Hepatocytes were isolated form Sprague-Dawley rats weighing 250-300g that had free access to laboratory chow and water. Rats were anesthetized with diethylether at 10 AM. L_4-hepatocytes were isolated by the two-step collagenase perfusion technique [26-27] with slight modifications. In brief, the inferior vena cava just above the kidney was ligated, the inferior vena cava above the diaphragm and the hepatic portal vein were cannulated and the liver perfused in situ for 20 min via the portal vein by Ca^{2+}, Mg^{2+} -free Krebs-Ringer bicarbonate (KRB) buffer (NaCl 118 mM, KCl 5 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 24 mM, glucose 5.6 mM) containing 2 mM EDTA in nonrecirculatory manner to disrupt the desmosomes.[27] Since EDTA inhibits collagenase activity in the subsequent perfusion,[28] perfusion continued for 5-10 min by the same solution but without EDTA. This removed EDTA from the liver tissue.[28] During this step, the liver was removed and perfused by KRB without calcium chelator but containing collagenase (200 U/mL) in the recirculatory system for 10 min.[28,29] At this step the liver became very soft and liver residue was run back and forth gently in the latter solution for 5-10 min at 37° C. Glisson's capsule was removed from the liver and the cell suspension was filtered through a 100 μm stainless-steel mesh to remove clumps and undigested tissue. The cell suspension was centrifuged at 1500 g for 3 min and washed three times by KRB (containing 5.6 mM glucose). Dispersion and washing media were collected and analyzed for LDH activity. Trypan blue exclusion was greater than 90%, and less than 10% of total LDH was released into the dispersion and washing medium.

Hepatocyte incubation

Hepatocytes (8± 0.5 mg protein/mL) were incubated at 37°C in a total volume of 4 mL of KRB containing 0.5% (W/V) bovine serum albumin (fatty acid free), 0.25 mM oleate, 20 mM glucose and 2.5 mM $CaCl_2$ in siliconized flasks with rubber stoppers shaking at 90 cycles/min under an atmosphere of O_2:CO_2 (19:1).

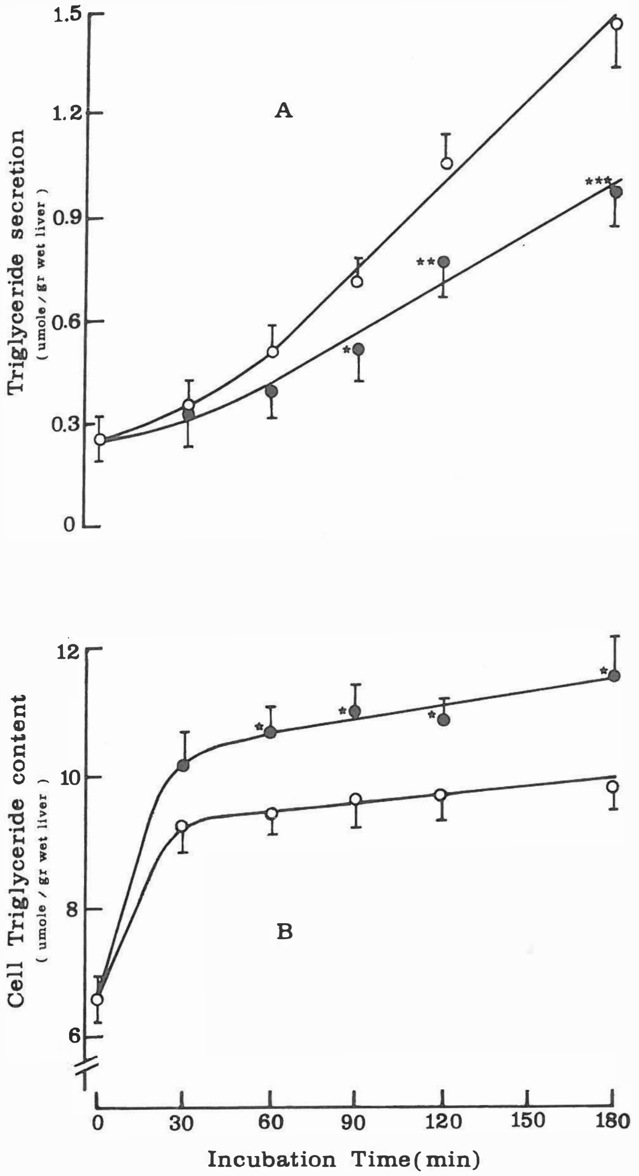

Fig. 2. Timecourse of triacylglycerol secretion (A) and hepatocyte triacylglycerol content (B) in the absence (○) and presence (●) of epinephrine (10 μM). The mass of secreted triacylglycerol was measured in the lipid extract of the incubation medium. *, **, *** indicate that the corresponding value is significantly different from its respective control at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.005$ co
are similar to Fig. 1.

Lipid analyses

At the end, incubation was stopped on ice and the medium removed after centrifugation at 1500 g for 3 min and the cell pellet was washed three times by 2 mL of fresh medium (KRB containing 20 mM glucose). The medium was centrifuged at 12000 g for 10 min at 4°C to remove cell debris,[7] and aliquots of supernatant were taken for direct analysis of triacylglycerol. The supernatant was extracted for lipid analysis by chloroform: methanol (2:1) by the method of Folch.[30] The cell pellet was resuspended in ice cold hemogenization solution (225 mM sucrose, 1mM EDTA and 50 mM Tris HCl, pH=7.4) and was hemogenized for 10 seconds by ultrasonic processor in an ice cold surrounding.[33] A portion of the sonicated cells was taken for protein determination and 1 mL was extracted for lipid analysis. Since disrupted lysosomal enzymes can hydrolyse cellular triacylglycerol and fatty acids, the product of the reaction can cause turbidity in subsequent triacylglycerol analysis, therefore the hemogenization must be done in ice cold medium within the shortest time and extracted rapidly for lipid analysis.

Secretion of VLDL was estimated by measuring the appearance of triacylglycerol in the incubation medium as it is well established that more than 95% of medium triacylglycerol are in the form of VLDL.[18,31-33] Dependence of .nedium triacylglycerol into VLDL fraction was demonstrated by precipition and ultracentrifugation as dicribed by Mangiapane and Brindley.[33] The rate of triacylglycerol secretion ($\overline{V}_{r.TG}$) was expressed as μmole TG/h/g wet liver. It is assumed that one μmole of triacylglycerol is equal to 885 μg and one gram wet weight of liver is equal to 158 mg of total cell protein.[32] This unit can be converted to μgTG/h/mg cell protein by the factor 5.6.

The mass of triacylglycerol in the cell and medium was analyzed by an enzymatic kit (Triacylglycerol, GPO-PAP) obtained from Zist-Shimi Co., Iran.[34] Mass of total phospholipids was evaluated by measuring phosphorus in organic phase using the method of Bartlet.[34] Bovine serum albumin was defatted from fatty acid and phospholipids by iso-octane, acetic acid[35] and methanol.[36] Bovine serum albumin-oleate complex was prepared just before hepatocyte incubation by simply adding potassium oleate to the albumin solution.[33]

Other analytical procedures

Protein concentration was determined by the method of Lowry et al.[37] LDH was assayed by a colorimetric method using a diagnostic kit (Sigma).

Statistical analysis

The results were expressed as mean ± S.E.M. The statistical significance of any observed differences was tested by Student's t-test.

nfidence levels, respectively. A

RESULTS

The time course curve for triacylglycerol secretion in the presence and absence of epinephrine (10 μM) is presented in Fig. 1, in which the mass of triacylglycerol was measured in the incubation medium directly, without lipid extraction. The results show that in spite of low triacylglycerol concentrations, measurements are still reproducible. The slope of the control curve indicates the mean rate of triacylglycerol secretion and was approximately $\overline{V}_{r,TG}$= 0.77 µmole TG/h/g wet liver. Comparison of the slopes of the curves in the presence and absence of epinephrine demonstrated that epinephrine suppressed triacylglycerol secretion by about 33% (Fig. 1).

Similar experiments were done in which triacylglycerol concentrations were measured in the lipid extract of the incubation medium (Fig. 2A). The measurements were more difficult at the first hour of incubation, and hence the difference between control and epinephrine treated samples was statistically significant only after this period of incubation. The rate of triacylglycerol secretion was 1.47± 0.11 and 0.98±0.09 (µmole TG/3h/g wet weight of liver) in the control and epinephrine treated samples respectively, which indicates 33% inhibition ($p\leq0.005$).

Changes in the hepatocyte triacylglycerol level with respect to time is shown in Fig. 2B. A rapid significant rise of cellular triacylglycerol content was observed in the first 30 min of incubation in both control and epinephrine treated cells. In this period, the total triacylgycerol in the system (cellular and medium) increased by ≥45% which was essentially attributed to increasing cellular triacylglycerol content. This was due to the new high levels of glucose and oleate in the incubation medium relative to the perfusion medium, suggesting that triacylglycerol synthesis was stimulated. Furthermore, epinephrine caused an increment in cellular triacylglycerol content over time, such that the difference between cellular triacylglycerol in control and epinephrine treated cells was almost constant with respect to time. It seems that the system reached a quasi-steady state after the first 30 min and then the total triacylglycerol content of the system will be equal in the presence and absence of epinephrine at every time.

The time course of hepatocyte phospholipid content has been shown in Fig. 3. In the absence of epinephrine, cellular phospholipid remained constant during the early stages of the incubation and also thereafter. However, in the presence of epinephrine, cellular phospholipid is slightly higher than the respective control ($p\leq0.05$) at all points of time.

The dose-response relationship of the effects of epinephrine on triacylglycerol secretion and cellular triacylglycerol contents (Fig.4A,B) revealed that the biological response to epinephrine was achieved at epinephrine concentrations of more than 1 µM. The optimum hormone concentration was deduced to be 10 µM, in which the curve reaches a plateau. The secretion rate of triacylglycerol was reduced, whereas the cellular triacylglycerol content increased with increasing concentrations of epinephrine in the medium. The increment in cellular triacylglycerol reflected primarily or exclusively the block in triacylglycerol secretion in response to epinephrine, and hence the sum of the cell and medium

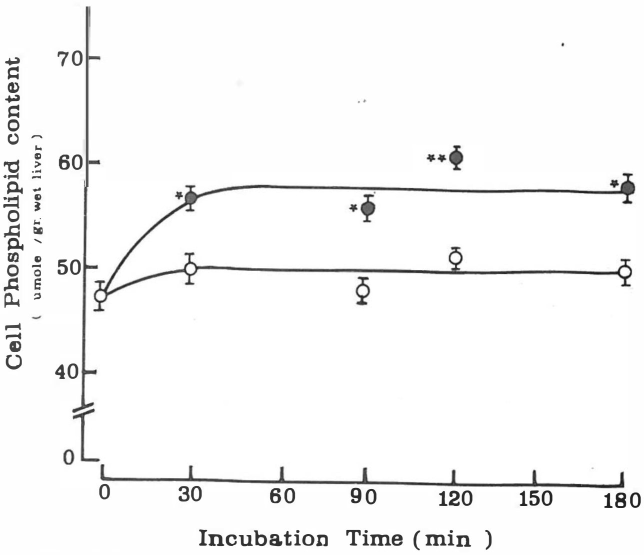

Fig. 3. Time course of hepatocyte phospholipid content in the absence (○) and presence (●) of epinephrine. The total mass of phospholipid was measured in the lipid extract of the cell as described in *Materials and Methods*. Each point represents the mean ± S.E. of four interassays in one experiment. The significance of the differences relative to control value is indicated by: *$p\leq0.05$ and **$p\leq0.025$.

triacylglycerol was almost constant at every concentration of epinephrine. Epinephrine at the maximum effective concentration (100 µM) suppressed triacylglycerol secretion by a maximum of 35% and increased cellular triacylglycerol content by about 20%.

The bioeffect of epinephrine is represented on the basis of 100% biological effect (Fig. 5), in which maximum inhibition of triacylglycerol secretion was chosen as 100% bioeffect. A similar curve could be plotted for cellular triacylglycerol content. This leads to a standard dose response curve and hormone concentration for 50% of the maximal bioeffect can be deduced more accurately, yielding an EC_{50} value of approx. 1 µM. The curve also demonstrated negative cooperativity of the epinephrine bioeffect. It is evident that at low concentrations of hormone [H], the bioeffect is linearly related to [H] whereas at greater concentrations of hormone the bioeffect is linear with log [H]. Negative cooperativity has been demonstrated for many hormones and provides exquisite sensitivity to low concentrations of hormones but protects the biological system against acute elevations of the effectors.

DISCUSSION

In the present study, triacylglycerol secretion rate was measured in isolated rat hepatocytes. The rate was comparable to those obtained by others[14-17,31-32] and to particulate phosphatidate phosphohydrolase total activity as described previously.[38-39] Triacylglycerol concentrations

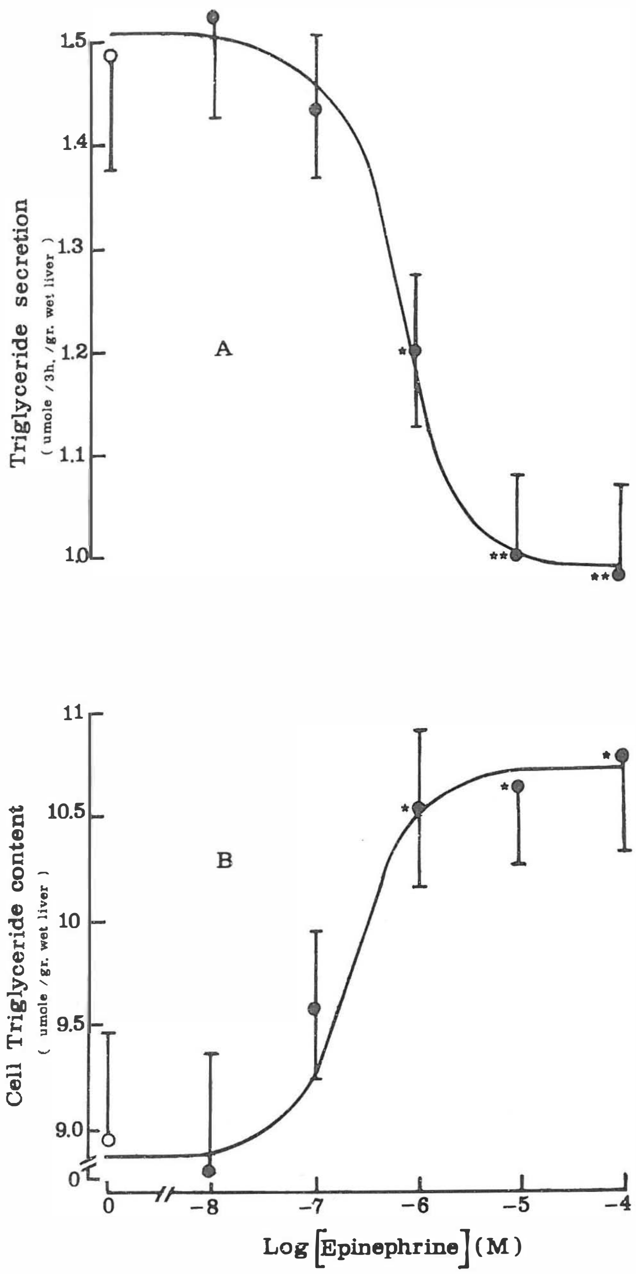

Fig. 4. Dose-response relationship of the effects of epinephrine on triacylglycerol secretion (A) and hepatocyte triacylglycerol content (B). Hepatocytes were incubated in KRB containing 20 mM glucose and 0.25 mM oleate for 3h at 37°C. The mass of triacylglycerol was measured in the lipid extract of the incubation medium and cells as described in *Materials and Methods*. The significance of the differences relative to control value is indicated by $*p \leq 0.05$ and $**p \leq 0.01$.

in the incubation medium depend on the cell concentrations, incubation time and concentrations of lipogenic precursors, being as low as 5±2 mg/dL in the first hour of incubation. Because of the low triacylglycerol concentration and the relative insensitivity of the triacylglycerol assay, it was more difficult to measure VLDL triacylglycerol secretion accurately during the first hour of incubation as described by Gibbons.[40] However, the time course of triacylglycerol secretion has been obtained in which the mass of secreted triacylglycerol has been measured by colorimetric [31] and fluorimetric methods.[14] We have also measured triacylglycerol directly without lipid extraction in aliquots of the incubation medium. Although triacylglycerol concentrations were very low, the absorbance was still completely linear even at 1.25 mg/dL of triacylglycerol. The results of the latter procedure were still reliable at the early periods of incubation. Results on the basis of triacylglycerol measurements with or without lipid extraction coincided, except that the triacylglycerol secretion rate on the latter basis exceeded the former (i.e. 2.31±0.22 versus 1.47±0.11 μmol TG/3h/g wet liver). Both procedures revealed that epinephrine inhibits triacylglycerol secretion by about 33%. Brindle and Ontko reported that epinephrine (10 μM) suppressed triacylglycerol secretion by about 50%.[14-15] The difference is probably related to the chosen molar ratio of nonesterified fatty acid to albumin, $\nu = [FA] / [Alb]$. The ratio determines the (unbound) free fatty acid level,[41] and both the ratio and concentrations are important.[42-43] These authors used the ratio: $\nu = 0.5/0.3 \simeq 1.7$, whereas in the present work the ratio was: $\nu = 0.25 / 0.075 \simeq 4$. It can be predicted that the effect of epinephrine will diminish by increasing the ν values and concentrations.[44]

Since the concentrations of glucose and oleate differ in the perfusion and incubation media, incubation is accompanied with new levels of them. Therefore, time course curves bear the effects of two variables, i.e. the effects of epinephrine and new levels of glucose and oleate. Cellular triacylglycerol content in both control and epinephrine treated cells increased markedly in the first 30 min of incubation and gradually thereafter. This is because of transferring the hepatocytes from perfusion medium (KRB containing 5.6 mM glucose without oleate) to the incubation medium (KRB containing 20 mM glucose and 0.25 mM oleate). The increment in cellular triacylglycerol has been reported in the presence of glucose [32] and extracellular fatty acids.[45] The glucose level was chosen identical and hyperglycemic in both perfusion and incubation media,[14-17,23,40] as the latter enhances viability of hepatocytes through preventing glycogenolysis and maintenance of the hepatocyte glycogen stores.[40] However due to observing this interesting phenomenon, glucose concentrations have been chosen differently in the perfusion and incubation media.

The most notable observation of the present investigation was a marked decrement in triacylglycerol secretion paralleled to an increment in its cellular content. The latter observation is not in agreement with the report of Brindle and Ontko.[14] The inverse relationship between the changes of the mass of triacylglycerol in the cell and incubation medium has been reported in the presence of insulin,[32]

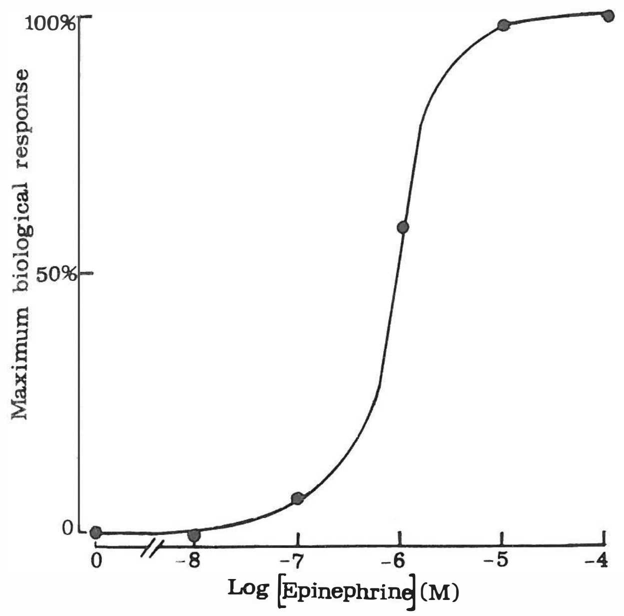

Fig. 5. Standard dose-response curve for epinephrine effect on triacylglycerol secretion on the basis of 100% bioeffect. The curve is deduced from Fig. 4A. Epinephrine concentration for half of the maximum effect is about 1 μM.

prostaglandins[17] and calcium antagonists,[18-19] and sulfonylureas[46] but not in epinephrine treated hepatocytes.[14] However, epinephrine inhibits lipogenesis[4] while prostaglandins have no effect[17] and cellular triacylglycerol accumulation is higher in the presence of insulin[32] in comparison with epinephrine. The mentioned inverse relationship was also observed in the presence of dexamethasone which enhances triacylglycerol secretion and decreases triacylglycerol and phosphatidylcholine remaining within the hepatocytes.[33] In the absence of epinephrine, cellular phospholipid in contrast to triacylglycerol remained constant during the incubation time. This means that cellular phospholipid levels do not depend on the concentration of glucose and fatty acid in the medium.[45] It is evident that the rapid accumulation of triacylglycerol in the hepatocyte suggests the importance of triacylglycerol as a storage energy but the lack of change in cellular phospholipids impress its structural rather than metabolic function. However, in the presence of epinephrine both cellular triacylglycerol and phospholipid increased slightly but still statistically significant. It is believed that nonreproducibility encountered in the assays of the metabolites covers the slight statistical difference which essentially exists. For instance, marked cell lysis can lead to high fatty acid levels which cause turbidity and interfere in triacylglycerol measurement.[47] This also exists for initial high fatty acid concentrations in the incubation medium.[47] In addition, the measured mass and the incorporation of radiolabelled substrate into metabolites will not always coincide.[32] The hypothesis that epinephrine has no effect on cell triacylglycerol levels may come from the fact that it has no effect on the incorporation of radiolabelled substrate to cellular triacylglycerol.[14] Furthermore, the incorporation of radiolabelled oleate into cellular phospholipid decreased in the presence of epinephrine,[17] while our results indicated an increment in the mass of cellular phospholipid, but these results do not contradict each other.

The increment in cellular cholesterol,[17,22] but not apolipoprotein-B,[8-11] along with the increasing of cellular triacylglycerol have also been reported in cultures of rat hepatocytes. The slight increments of cellular lipids do exist in the presence of epinephrine, but it never means "cellular lipid accumulation". Since the difference between cellular lipid content in the presence and absence of epinephrine is constant during the time course, the difference indicates only the prompt lipids that would not be secreted yet. It may be that triacylglycerol accumulating in the secretory pathway of epinephrine treated cells is subject to lipolysis. This would prevent excessive accumulation of triacylglycerol within hepatocytes.

Previous report indicated that epinephrine also suppresses pre-labeled triacylglycerol secretion from rat hepatocytes.[15] The effect of epinephrine on VLDL secretion is mediated via α_1-adrenoceptors.[15-16] Alpha-1 stimulation of hepatocytes is associated with a release of calcium from the endoplasmic reticulum (ER) to the cytosol.[24-25] An optimum concentration of Ca^{2+} within the ER is essential for assembly and secretion of VLDL.[8] Therefore, it seems that epinephrine exerts an inhibitory effect on VLDL secretion, probably through blocking in the secretory pathway.

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