Retinoic Acid Receptor Transcripts and Effects of Retinol and Retinoic Acid on Glucagon Secretion From Rat Islets and Glucagon-Secreting Cell Lines

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Using intact rat islets, hamster In-R1-G9 cells, and mouse αTC-1 clone 9 transgenic tumoral glucagon-secreting cells, we determined the effects of retinol (ROH) and retinoic acid (RA) on glucagon secretion. Since vitamin A effects may be mediated through nuclear RA receptors (RARs) and cytoplasmic ROH- and RA-binding proteins (CRBP and CRABP), cells were also assayed for RARs, CRBP, and CRABP mRNA by Northern blot analyses. Islets and cells were cultured in 2.8 mmol/L glucose and vitamin A–deficient (A-def) medium or in different concentrations of ROH and RA. Using intact islets, RA 10 and 100 nmol/L inhibited glucagon secretion to approximately 60% of control levels. Using In-R1-G9 cells, ROH 0.175 to 5.0 μmol/L inhibited glucagon secretion to 60% to 83% of control levels, and RA 100 and 1,000 nmol/L inhibited glucagon secretion from 72% to 43% of control levels, respectively. Using αTC-1 cells, ROH 1.75 μmol/L inhibited glucagon secretion to 80% of control levels, and RA 1 to 100 nmol/L inhibited secretion from 83% to 68% of control levels. Inhibition of secretion was dose-dependent. RARα RNA transcripts were detected in αTC-1 and In-R1-G9 total RNA extracts; RARγ transcripts were detected in αTC-1 cells. We conclude the following: (1) ROH and RA inhibit glucagon secretion in cultured rat islets and glucagon-secreting cell lines, and in cell lines the effect of RA is dose-dependent; (2) on a molar basis, RA is on the order of 10- to 100-fold more potent than ROH, a finding consistent with RA being the active metabolite of ROH at the α-cell level; and (3) this inhibition may be mediated through classic pathways of retinoid action involving nuclear RARs and gene expression of specific proteins.

VITAMIN A (retinol [ROH]) is required for vision and for reproduction, growth, and differentiation of a variety of cells.[1] Retinoic acid (RA) can substitute for ROH in growth and differentiation of cells, and at high doses in reproduction.[2,3] However, RA cannot substitute for ROH in vision.[2] With the exception of the role of vitamin A in vision,[4] molecular mechanisms of vitamin A action have been elucidated only recently. Intracellularly, ROH and RA bind to specific cytosolic ROH- and RA-binding proteins (CRBP and CRBP II and CRABP and CRABP II, respectively)[5,6] and nuclear RA receptors (RXR and RAR α, β, and γ).[6,7] The functions of CRBP and CRABP and other more recently identified cytoplasmic binding proteins[5] are not known, but they may serve to regulate the availability and concentration of intracellular ROH and RA by transporting or shuttling retinoids between subcellular organelles, solubilizing intracellular retinoids, serving as a readily available storage pool, or targeting retinoids to specific metabolic pathways.[5]

Two families of nuclear receptors, each consisting of three classes referred to as RAR and RXR α, β, and γ, have been identified in embryonic and adult tissues. RAR and RXR α, β, and γ originate from different genes, and each RAR gene encodes isoforms that are generated by differential promoter usage, alternative splicing, or both mechanisms.[6,7] RAR binds to RA or to 9 *cis*-RA and didehydroretinoic acid, metabolites of RA, whereas RXR binds to 9 *cis*-RA only. These nuclear receptors belong to a superfamily of nuclear receptors that include receptors for steroids and thyroid hormone[7]; these receptors are ligand-binding transcription factors that mediate hormone effects through gene expression.[6,7] RARs and RXRs, either alone or through dimerization with each other or other transcription factors, may mediate the effects of RA on growth and differentiation through regulation of gene expression.[6-8] Since ROH is metabolized intracellularly, nuclear receptors mediate the effects of ROH indirectly after intracellular conversion of ROH to RA, 9-*cis* RA, or didehydroretinoic acid.[7]

We reported that ROH is required for insulin secretion in vivo, and that RA could substitute for ROH in this requirement.[9] In our efforts to define the cellular mechanism of vitamin A action, we demonstrated RAR α and γ transcripts and CRBP and CRABP transcripts and proteins in insulin-secreting cells.[10-12] In the latter studies, CRBP and CRABP were found also in non-β cells.[10,11] More recently, we showed that vitamin A deficiency in the rat leads to a defect in glucagon secretion that may be irreversible.[13] Therefore, to test for the direct effects of vitamin A on the α cell, we tested for the effects of ROH and RA after 48 hours of culture on glucagon secretion using tumoral mouse αTC-1 transgenic cells, hamster In-R1-G9 glucagon-secreting cells, and intact rat islets. Since vitamin A effects may be mediated through RARs, cells were also assayed for the presence of mRNA for RAR α1 (formerly α0), RAR β2 (formerly β0), and RAR γ_a. Our studies reported here show that RA inhibits glucagon secretion from glucagon-secreting cells and islets in culture.

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MATERIALS AND METHODS

Rat Islet Isolation and Culture

Rat pancreases were removed from normal Sprague-Dawley rats, and islets were isolated by methods previously described.[12] Islets were washed twice by repetitive transfers into a clean dish containing culture media. After the second washing, batches of 10 islets were transferred into wells of a multiwell culture plate containing 1 mL culture media without (A-def) or with RA 1, 10, or 100 nmol/L. The islets were cultured at 37°C with 5% CO_2 in a humidified incubator. After 48 hours, media were removed and replaced with Krebs-Ringer buffer containing 2.8 mmol/L glucose, and the islets were allowed to incubate for a 1-hour equilibration period. Then the media were replaced with Krebs-Ringer buffer containing either 2.8 mmol/L glucose or 2.8 mmol/L glucose plus arginine 19 mmol/L and islets were incubated for a test period of 1 hour, after which the media were collected and assayed for glucagon.

αTC-1 and In-R1-G9 Cell Lines and Culture

In-R1-G9 and αTC-1 clone 9 cell lines were kindly provided by Dr Junko Ono (First Department of Medicine, Medical College of Oita, Oita, Japan) and Dr K. Hamaguchi (The Jackson Laboratory, Bar Harbor, ME), respectively. The glucagon-secreting In-R1-G9 clone was derived from parent BK virus–transformed insulinoma cells (In-111-R1) of a Syrian hamster.[14,15] The glucagon-secreting αTC-1 clone 9 cell line was derived from parent cells of an adenoma produced in transgenic mice.[16] αTC-1 cells were cultured at 37°C in 5% CO_2/air in flasks containing Dulbecco's modified Eagle's medium supplemented with glucose 16.7 mmol/L, HEPES 15 mmol/L, sodium bicarbonate 44 mmol/L, nonessential amino acids, 0.02% bovine serum albumin, 10% heat-inactivated fetal bovine serum, penicillin, and gentamicin. In-R1-G9 cells were cultured in RPMI 1640 medium containing glucose 11.1 mmol/L, HEPES 15 mmol/L, 5% heat-inactivated fetal bovine serum, penicillin, and streptomycin. Cultures were maintained in 5% CO_2 at 37°C. Medium was changed three times per week. αTC-1 cells grew to near-confluence and In-R1-G9 cells to confluence between days 5 and 7; then cells were dislodged from the surface using enzyme-free phosphate-buffered saline–based cell dissociation buffer (GIBCO-BRL, Gaithersburg, MD) or trypsin 0.05% and EDTA 0.02%, respectively. Cells were pelleted, the supernatant was discarded, and the pellet was snap-frozen in liquid nitrogen and stored at −70°C for subsequent isolation of total cellular RNA.

Measurement of Glucagon Release

Cells (0.5×10^6) were seeded into 12-well plates with a 22.6-mm well diameter and a 4-cm^2 growth area (Costar, Cambridge, MA). Experiments were designed to test for the effects of ROH and RA on secretion after a 48-hour exposure. The concentration of glucose in media during experiments was 2.8 mmol/L, a concentration that is in the low-physiological range, has little effect if any on basal secretion from dispersed glucagon-secreting cells, and does not interfere with the response to secretagogues.[14,16]

In experiments testing the effects of exposure to ROH and RA for 48 hours, cells were cultured in medium containing glucose 2.8 mmol/L, ROH 0.175, 1.5, 1.75, 3.0, and 5.0 μmol/L, or RA 1, 10, 100, and 1,000 nmol/L. Control cells were incubated in parallel in the same medium without ROH or RA. Ethanol, the vehicle for ROH and RA, was again added to control medium in a final concentration of 0.01%. The incubation medium was the same as the culture medium described earlier, except the fetal bovine serum was exposed to UV light (365-nm, 100W bulb, model B-100A UV lamp; UVP, San Gabriel, CA) for 8 hours to deplete retinoids.[17] After 48 hours of culture, media were removed and the cells were washed twice and incubated for 1 hour in Krebs-Ringer buffer containing 2.8 mmol/L glucose without ROH and RA. At the end of this hour, the medium was removed and centrifuged (150× g for 10 minutes). The supernatant was frozen for subsequent assay of glucagon by radioimmunoassay. Glucagon was assayed using G-15 antiglucagon antibody produced in rabbit, and a goat antirabbit serum as the second precipitating antibody.[18] G-15 antibody was provided by Dr Jonathan Jaspan (Tulane University School of Medicine, New Orleans, LA). Monoiodinated glucagon was kindly provided by Dr Bruce Frank (Lilly Research Laboratories, Indianapolis, IN). Assay sensitivity is 7.8 pg/mL, with an intraassay coefficient of variation of 7.5% and an interassay coefficient of variation of 8.3%.

Isolation of Total RNA

Total RNA was extracted from 10^6 plated cells by the method of Chirgwin et al.[21] Total RNA from liver and other tissues was prepared for Northern blot analyses as previously described.[22,23]

Northern Blot Analyses for RAR α, β, and γ

Total RNA was denatured in glyoxal and dimethyl sulfoxide, fractionated by electrophoresis on 1.2% agarose gel, and transferred to nylon filters. Mouse RAR cDNA clones were kindly provided by Dr Pierre Chambon (Institute de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France): RAR α1 (formerly α0), 2.1-kb cDNA inserted into the *Eco*RI site of pSG5 vector; RAR β2 (formerly β0), 1.95-kb cDNA inserted into the *Eco*RI site of pTZ19R vector; and RAR γA, 2.0-kb cDNA inserted into the *Eco*RI site of pTZ19R vector. Full-length RAR α and γ cDNAs were obtained for probe synthesis by digestion of RAR α and γ vectors with *Eco*RI. A nearly full-length 1.83-kb fragment of RAR β was obtained by digestion of pTZ19R with *Bam*HI and *Eag*I and used to synthesize a probe. ^{32}P-radiolabeled probes for Northern blots were prepared by random oligonucleotide priming of specific gel-purified DNA fragments.[24] Hybridizations with cDNA probes were performed at 42°C overnight. Blots were washed in 2× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate) and 0.1% sodium dodecyl sulfate at room temperature for 20 minutes and 0.2× SSC and 0.1% sodium dodecyl sulfate at 50°C for 20 minutes. Blots were exposed for 5 to 14 days at −70°C using an intensifying screen and Kodak XAR-5 film (Eastman Kodak, Rochester, NY). In all Northern blots, rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA[25] was used as an internal standard for RNA quantity and integrity. Commercially available RNA ladders were used as molecular-weight markers; these markers were visualized by ethidium bromide staining.

Statistical Analysis

Statistical significance of differences between means of multiple groups was tested by ANOVA and Student-Neuman-Keuls and least-significant difference tests.[19,20] The value for secretion from cells or islets cultured in a single well was considered as $n = 1$ for statistical analyses. Cell secretion results in most studies represent secretion from cells from different passages cultured on different days. Values are expressed as the mean ± SEM.

RESULTS

Effects of RA on Glucagon Secretion From Cultured Islets

Under the conditions of the experiment, arginine did not significantly stimulate glucagon secretion (Table 1). Data

Table 1. Effects of RA on Glucagon Secretion (pg/islet) From Rat Islets Cultured in Glucose Alone or Glucose Plus Arginine

RA (nmol/L)	Glucose (2.8 mmol/L)	Glucose (2.8 mmol/L) + Arginine (19.0 mmol/L)
0	89.2 ± 10.9	78.8 ± 7.2
1	76.7 ± 16.6	64.3 ± 6.3
10	62.3 ± 7.7	43.4 ± 9.7
100	62.1 ± 9.7	49.3 ± 8.0

NOTE. Arginine did not stimulate secretion, and data from the two treatments at different RA concentrations were combined and are shown in Fig. 1. n = 6 for all groups except glucose + arginine at RA 10 nmol/L, where n = 5.

from control and arginine-stimulated experiments were combined. RA inhibited glucagon secretion to approximately 60% of secretion from islets cultured in A-def media (Fig 1).

Effects of ROH and RA on Glucagon Release From Cultured Cells

To determine the effect of culture in ROH and RA for 48 hours, secretion during the first hour was expressed as a percent of time-matched secretion from control cells not cultured in ROH or RA. ROH 1.75 μmol/L inhibited secretion from In-R1-G9 cells (Fig 2) to 61% of control levels ($P < .01$). A consistent dose-response effect was not observed. Although ROH 1.75 μmol/L appeared to inhibit glucagon secretion more than ROH 0.175 and 1.5 μmol/L, higher concentrations of ROH, 3 and 5 μmol/L, did not further inhibit glucagon secretion and had effects similar to 0.175 and 1.5 μmol/L. RA 100 and 1,000 nmol/L inhibited secretion to 72% and 43% of control levels, respectively

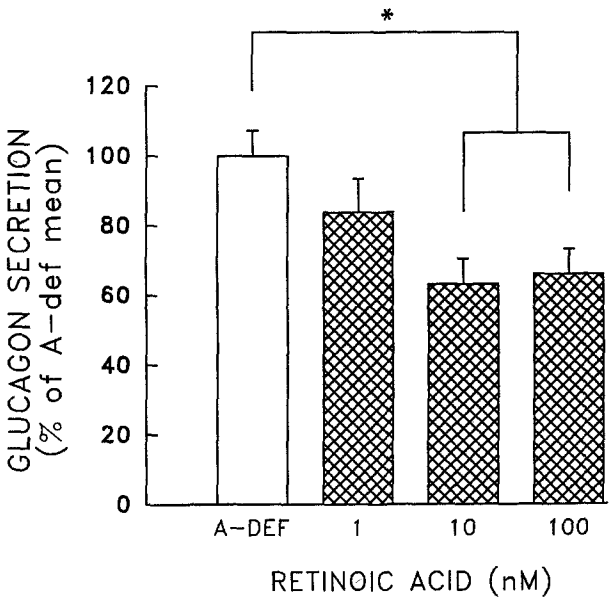

Fig 1. Effects of RA on glucagon secretion from rat islets cultured in 2.8 mmol/L glucose. Islets were cultured in RA 1, 10, and 100 nmol/L for 1 hour. Control secretion from islets cultured in A-def media was 84 ± 6.4 pg/islet and normalized to 100%. RA inhibited glucagon secretion ($P < .01$). *$P < .01$, A-def *v* RA 10 and 100 nmol/L. n = 10 to 11 for all groups.

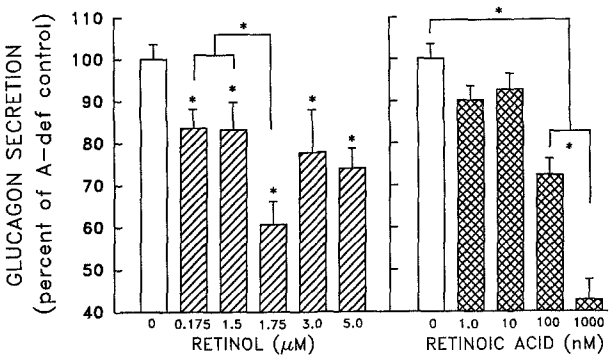

Fig 2. Effects of a 48-hour culture in different concentrations of ROH and RA on basal glucagon secretion from In-R1-G9 cells during a 1-hour test period. Following exposure to ROH and RA, media were changed to include 2.8 mmol/L glucose and cells were incubated for 1 hour. Control secretion was 4,617 ± 459 pg/10^6 cells. ROH and RA inhibited glucagon secretion ($P < .001$). Differences were significant at $P < .05$ for the following: A-def *v* ROH at each concentration, ROH 0.175 *v* 1.75 μmol/L, ROH 1.5 *v* 1.75 μmol/L, A-def *v* RA 100 and 1,000 nmol/L, and RA 100 *v* 1,000 nmol/L. n = 60 for A-def and 17 to 42 for other groups. *$P < .05$.

($P < .05$). ROH and RA also inhibited secretion from αTC-1 cells (Fig 3). ROH 1.75 μmol/L inhibited secretion to 86% of control levels ($P < .05$), and RA 10 and 100 nmol/L inhibited secretion to 76% and 68%, respectively ($P < .05$). The effect of RA was concentration-dependent ($P < .05$) in both cell lines.

Northern Blot Analyses for mRNA for RAR α, β, and γ

After exposures for 2 and 5 days, blots of αTC-1 cell RNA showed two transcripts for RAR α and one for RAR γ (Fig 4). RAR α transcripts were 3.5 and 2.6 kb, respectively. The RAR γ transcript was approximately 3.2 kb. Blots of In-R1-G9 cell RNA showed two α transcripts and one γ transcript. The GAPDH transcript was 1.4 kb, evident after exposure times as short as 20 minutes. The

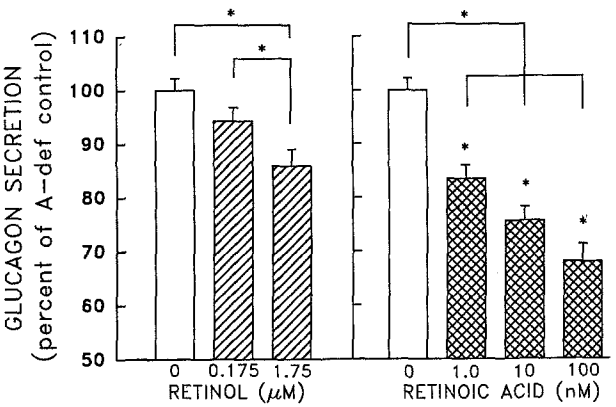

Fig 3. Effects of a 48-hour culture in different concentrations of ROH and RA on basal glucagon secretion from αTC-1 cells during a 1-hour test period. Design of the experiment was the same as in Fig 2. Control secretion was 4,789 ± 814 pg/10^6 cells. Both ROH and RA inhibited glucagon secretion ($P < .001$). n = 28 to 30. Differences were significant at $P < .05$ for the following: control *v* ROH 1.75 μmol/L, ROH 0.175 *v* 1.75 μmol/L, control *v* each RA concentration, RA 1.0 *v* 10 nmol/L, RA 1.0 *v* 100 nmol/L, and RA 10 *v* 100 nmol/L. *$P < .05$.

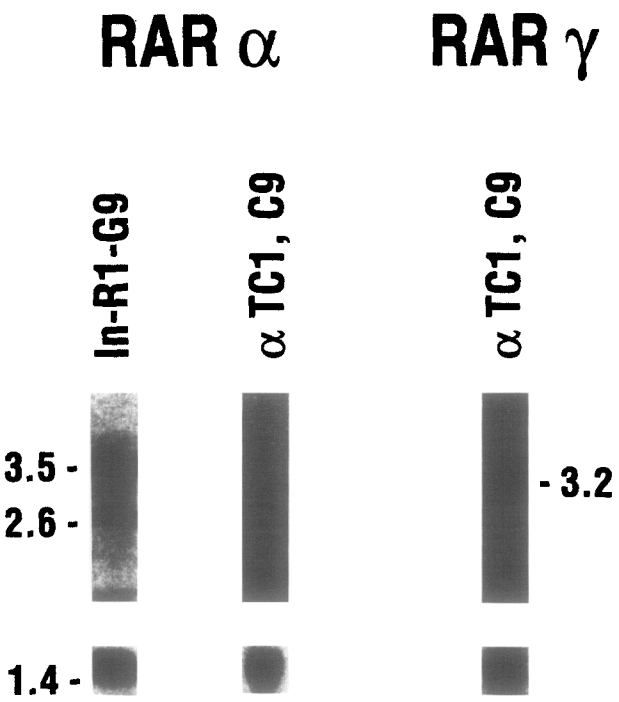

Fig 4. Northern blot analyses of αTC-1 and In-R1-G9 cell total RNA for RAR α and RAR γ. Two RAR α bands were evident at 3.5 and 2.6 kb in both In-R1-G9 and αTC-1, C9 cells, although they were of lesser intensity in the latter line. A single RAR γ band was present at 3.2 kb in αTC-1 cells. GAPDH bands migrating at 1.4 kb are shown at the bottom of each lane. *$P < .05$.

5-day exposure of the blot for RAR β showed nonspecific hybridization to ribosomal RNA and no detectable RAR β transcript. Repeated blots including those with prolonged exposure times for 3 weeks failed to detect RAR β transcripts in αTC-1 and In-R1-G9 cell extracts.

DISCUSSION

In previous studies using islets and insulin-secreting cells, we identified cytosolic retinoid-binding proteins and nuclear RARs[9-12] and demonstrated impaired insulin release from islets of A-def rats[9] and insulin-secreting cells in the absence of RA.[10] These findings suggested a role for retinoids in insulin secretion at the islet and β-cell level.

Further studies exploring for possible effects of retinoids on the α cell, as well as the β cell, showed impaired glucagon release from islets of A-def rats and the presence of CRBP and CRABP in In-R1-G9 cells.[13] The identification of RARs in glucagon-secreting cells reported here further supports an effect of RA on the α cell. However, the inhibitory effect of RA on glucagon secretion from glucagon-secreting cells was unexpected. Based on our previous finding of defects in glucagon secretion from islets of A-def rats,[13] we expected secretion to be decreased in the absence of vitamin A in vitro and repletion of media with RA to improve secretion. Instead, RA inhibited glucagon secretion from islets and both cell lines. We would conclude that findings in vivo in the A-def rat may not be germane to secretion studies in vitro. There are several possible explanations for this difference. (1) Studies in vivo may reflect different effects of retinoids. In animal studies, rats develop vitamin A deficiency over 60 days and have signs of vitamin A deficiency for at least 2 weeks before study. Based on the irreversibility of the defect in glucagon secretion with vitamin A repletion, we suggested that vitamin A deficiency may influence early islet development and function.[13] (2) Prolonged deficiency may lead to adverse effects on tissues and production of other modulators that decrease glucagon synthesis or secretion. (3) Rats with prolonged deficiency may actually be chemically deficient in ROH and metabolites of ROH other than RA. Thus, the suppression of glucagon in vitro may actually reflect acute or more physiological functions of RA compared with effects observed in the A-def state developed over 60 days.

These effects on secretion after 48 hours are probably mediated through RARs and gene expression.[8] These inhibitory effects of retinoids after culture for 48 hours are probably important biologically, since vitamin A effects on growth and differentiation are thought to be mediated through gene expression (see below).

On a molar basis, RA was 10 to 100 times more potent than ROH. However, ROH is present in a higher concentration than RA in serum (ROH ~1.5 to 2 μmol/L *v* RA ~5 to 10 nmol/L), so the concentrations of ROH we selected to test were within the physiological range,[6] and these effects could be physiologically important. RA, not ROH, is thought to be the biologically active metabolite, with ROH being converted to RA intracellularly. Circulating ROH in micromolar concentrations may give rise to concentrations of intracellular RA equal to or greater than the concentration of RA we tested, and this may account for the observed effect of ROH.

Differences in the effects of RA on secretion between islets and cell lines and between cell lines were observed. These differences may be related to transformation and the amounts of intracellular RAR, CRBP, and CRABP. For example, islets were sensitive to 10 nmol/L RA, whereas cultured cells responded to higher concentrations of RA in a concentration-dependent manner; supraphysiological concentrations of RA, ie, 10^{-6}, caused maximal effects. Since tumoral cells are transformed, unlike islets, they may be resistant to low concentrations of RA. αTC-1 cell secretion was inhibited to a greater extent than that observed for In-R1-G9 cells. These cell line–dependent effects of RA on secretion may be related to the ability of each cell line to take up and metabolize ROH and RA, or to the concentrations of CRBP, CRABP, and RARs. Tissues and cell lines differ in amounts of RARs, CRBP, and CRABP.[5,7,26,27] These binding proteins probably serve different functions in mediating the actions of their respective ligands. CRBP has been proposed to control the availability of ROH for cells, generating high levels of RA through oxidation of ROH, and is coincidentally distributed with the expression of RAR β.[26] CRABP regulates the amount of free RA available, establishes RA gradients, and may transfer RA to the nucleus.[27] RARs mediate the effects of vitamin A either directly or indirectly on the ensuing expression of over 300 genes and their protein products.[5,11] Some of these vitamin

A–dependent products are directly or indirectly related to hormone secretion, eg, glucokinase[28] and transglutaminase.[29] Cytosolic retinoid-binding proteins and RARs may have important roles in mediating the effects of ROH and RA on αTC-1 and In-R1-G9 cell growth, differentiation, and secretion. Comparisons of relative concentrations of RARs, CRBP, and CRABP among glucagon-secreting cell lines may provide insight into their growth and secretion. It is possible that differences in secretion between αTC-1 and In-R1-G9 cells observed in our studies are related to qualitative or quantitative differences between cell lines in intracellular uptake or metabolism of ROH and RA, concentrations of RARs, CRBP, and CRABP, and binding of ROH or RA. This may result in more or less available intracellular ROH or RA, and, in turn, different responses. Cells without CRBP may not be able to accumulate ROH and convert it to RA, the active metabolite, or cells without CRABP may not be able to bind intracellular RA and thereby be sensitive to low concentrations of RA. Cells with low levels of or altered RARs may be relatively resistant to RA.

In these experiments, arginine failed to stimulate glucagon release. Although arginine and a low glucose concentration are known to stimulate glucagon secretion in vivo and in vitro from the perfused pancreas, these findings are inconsistently observed using collagenase-isolated islets in culture.[30,31] This may be a result of collagenase isolation or culture conditions. It is possible that the conditions, such as culture in 2.8 mmol/L glucose for 48 hours, may have reduced the glucagon response to arginine sometimes observed following culture in 11.0 mmol/L glucose.[31] Alternatively, UV irradiation of media to deplete endogenous retinoids could conceivably lead to inactivation of a component in the media, eg, phospholipids or other vitamins such as vitamin D metabolites,[32] that may be required for arginine-induced release.

Our current study showing that ROH and RA decrease glucagon secretion from αTC-1 and In-R1-G9 cells supports the idea that retinoids have a role in glucagon secretion. αTC-1 and In-R1-G9 models should be helpful in studying the cellular mechanisms by which retinoids influence glucagon secretion, with an effort made to identify target genes and protein products involved in secretion. Further studies on the effects of retinoids, their cytosolic binding proteins, and their nuclear receptors on glucagon secretion and phenotypic growth expression of glucagon-secreting cell lines would provide insight into the mechanism of action of retinoids in islet cell differentiation and growth and glucagon secretion. This knowledge may be applicable to the growth and development of normal islets and transplanted islets and cells. The inhibitory effect on glucagon secretion is of particular interest with regard to glucose homeostasis, since RA is known to stimulate insulin release. This diverse effect of RA on insulin secretion and glucagon secretion raises the possibility that dietary vitamin A and RA could be important in the bihormonal control of metabolism.

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