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Retinoic Acid and Triiodothyronine Stimulate ADP-Ribosyl Cyclase Activity in Rat Vascular Smooth Muscle Cells

Frederico G. S. de Toledo, Jingfei Cheng, and Thomas P. Dousa[1]

Renal Pathophysiology Laboratory, Department of Physiology and Biophysics, Mayo Clinic and Foundation, Mayo Medical School, Rochester, Minnesota 55905

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Cyclic ADP-ribose (cADPR) is a nucleotide synthesized from β-NAD$^+$ that can trigger or facilitate Ca^{2+}-release through ryanodine-channels. We investigated the synthesis of cADPR (ADPR-cyclase activity) in cultured vascular smooth muscle cells (VSMC) from rat aorta in response to incubation with all-*trans*-retinoic acid (RA), 3,3′,5′-triiodothyronine (T_3), cortisol, β-estradiol and 1-dehydrotestosterone. Only RA and T_3 caused concentration-dependent ($10^{-9} - 10^{-6}$ M) stimulation of ADPR-cyclase activity in VSMC. Maximum stimulatory responses to RA (+100%) and T_3 (+40%) were additive and the stimulatory effects of both hormones on ADPR-cyclase were due to an increase in Vmax without changes in the apparent Km. These observations indicate that in VSMC synthesis of cADPR can be upregulated by RA and T_3. We propose that some of the actions of RA on VSMC such as enhancement of contractile competence, differentiation, and anti-proliferative effects might be elicited, at least in part, via upregulation of the cADPR/Ca^{2+}-release signaling system. © 1997 Academic Press

The release of Ca^{2+} from intracellular stores (endoplasmic/sarcoplasmic reticulum) and consequent increase in cytoplasmic Ca^{2+} is a widespread signaling mechanism that regulates numerous functions in eukaryotic cells. Ca^{2+} is released by two types of channels (1,2). IP_3-sensitive channels respond to the well-established second messenger inositol-1,4,5-triphosphate (IP_3). Another route of Ca^{2+}-release is through ryanodine-sensitive channels (RyR) which can be activated in response to an increase in cytoplasmic Ca^{2+}-concentration, thus mediating the so-called Ca^{2+}-induced Ca^{2+}-release.

Cyclic ADP-ribose (cADPR) is a novel nucleotide that can trigger or facilitate Ca^{2+}-release through RyR or RyR-like channels, without effects on the IP_3-sensitive channel (3,4). Biosynthesis of cADPR from β-NAD$^+$ is catalyzed by enzymes with ADP-ribosyl cyclase activity (ADPR cyclase) and hydrolyzed to ADPR (non-cyclic and devoid of Ca^{2+}-release activity) by enzymes with cADPR-hydrolase activity (3,4). Enzymes of cADPR metabolism are ubiquitous in mammalian cells (3) and a novel concept is emerging which posits that cADPR-triggered or modulated Ca^{2+}-release plays a regulatory role as a second messenger in signal transduction (4).

Unlike in the case of the well-established IP_3/Ca^{2+}-release system, the regulation of cADPR metabolism in signal transduction remains little explored. A stimulatory effect of retinoic acid (RA) upon ADPR-cyclase was found in renal LLC-PK_1 cells (5), and also in promyelocytic HL-60 cells (6,7) and THP-1 cells (6). In a recent study (8), we observed that vascular smooth muscle cells (VSMC) from rat aorta grown in primary culture have high ADPR-cyclase activity and we subsequently found that *in vivo* administration of β-estradiol to rats markedly increases ADPR-cyclase activity and cADPR generation specifically in myometrium but not in liver, skeletal muscle or brain (9). In intestinal longitudinal smooth muscle cells, cADPR was reported to trigger Ca^{2+}-release and cADPR synthesis could be modulated by cholecystokinin octapeptide (10).

It is generally acknowledged that RA is a potent regulatory agent that controls cell growth and differentiation (11,12) and also promotes specific cell functions in VSMC such as contractility (13) and synthesis of elastin (14). Several studies indicate that RA suppresses key processes related to atherogenesis and vascular hypertrophy in VSMC (12,13,15).

In view of these considerations, we decided to investigate the effects of RA on ADPR-cyclase of VSMC. In addition, we also examined the effects of T_3 on ADPR-cyclase as nuclear receptors for RA and T_3 are similar

[1] Correspondence and reprint requests to: 921B Guggenheim Building, Mayo Clinic, Rochester, MN 5505. Fax: (507) 266-4710.

Abbreviations: cyclic ADP-ribose (cADPR); ADP-ribosyl cyclase (ADPR cyclase); 1-dehydrotestosterone (DHT); nicotinamide adenine dinucleotide (NAD); nicotinamide guanine dinucleotide (NGD); Ryanodine receptor (RyR); retinoic acid (RA); retinoic acid receptor (RAR, RXR); 3,3′,5′-triiodothyronine (T_3); T_3 receptor (T_3R).

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in structure and have been documented to bind in some instances to common hormone-responsive elements (16,17), suggesting that RA and T_3 may co-regulate gene expression.

MATERIALS AND METHODS

Vascular smooth muscle cell isolation and culture. Vascular smooth muscle cells (VSMC) were isolated and subcultured from rat aorta explant outgrowths as previously described (8). VSMC were characterized as previously described by positive immunocytochemical staining against α-actin and phase-contrast microscopy criteria (8,18). Briefly, aorta was isolated from 200-250 g male Sprague-Dawley rats, cleaned in ice-cold sterile 0.9% NaCl, endothelium removed by scrapping and adventicia removed with surgical tweezers. Tissue was then minced and placed on 100 mm Petri dishes with a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 nutrient mixture (DMEM/F-12) supplemented with 10% bovine fetal calf serum, 100 U/L penicillin, 100 μg/L streptomycin, and 0.25 μg/L amphotericin B. Cell cultures were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. After reaching confluence on 100 mm-diameter dishes, cells were washed twice with phosphate-buffered saline (PBS) and incubated in serum-free DMEM/F-12 without phenol red. After a 48 h period, fresh medium was replaced and hormones added. Cell were used between passages 4-19.

Preparation of membrane fraction. After 48 h with test hormones, cells were washed twice with 4 ml of ice-cold PBS and then lifted with a plastic cell scrapper in an ice-cold buffer containing 40 mM Tris-HCl (pH 7.2) and 0.25 M sucrose. Cells were then disrupted by sonication (3 cycles of 10 seconds each, 8 microns amplitude), and centrifuged at 2,000 g for 10 min. Supernatant was then centrifuged at 40,000 g for 30 min (9) and the resulting pellet resuspended in 0.4 ml of the same buffer described above. Protein content was measured by the method of Lowry et al (19).

Fluorimetric determination of ADPR-cyclase activity. The ADPR-cyclase activity measurements were based on the conversion of the NAD analog nicotinamide guanine dinucleotide (NGD^+) to the fluorescent product cyclic GDP-ribose (cGDPR), was continuously monitored at 300 nm excitation wavelength and 410 nm emission wavelength using a Hitachi F-2000 spectrofluorimeter. Enzymatic activity was calculated taking the initial linear slope steepness and the change in fluorescence calibrated to a standard curve with known concentrations of cGDPR (9,20). All experiments were conducted in 2-3 replicates and each replicate measured at least twice. Total membrane fraction (0.1 mg protein/ml) was incubated in the same buffer as described above and reaction was started by addition of 0.4 mM NGD.

Enzymatic synthesis of cADPR from β-NAD^+. Membranes (0.25 mg protein/ml) were incubated in a medium containing 40 mM Tris-HCl (pH 7.2), 0.25 M sucrose and 1 mM β-NAD^+ at 37°C with continuous shaking. 5-7 ml aliquots were drawn at specific times and tested for Ca^{2+} releasing activity using the sea urchin egg homogenate bioassay (5,8,9). In each experiment, authenticity of cADPR in aliquots was confirmed by blocking Ca^{2+}-release activity with 4 μM 8-Br-cADPR, a specific inhibitor of cADPR-mediated Ca^{2+}-release (5,8,9).

Sea urchin egg homogenate Ca^{2+} release bioassay. Homogenates from sea urchin eggs (*Lytechinus pictus*) were prepared as described (5,8,9). Fluorescence of Ca^{2+} probe fluo-3 was monitored at 490 nm excitation and 535 emission in a 0.25 ml cuvette at 17°C with circulating water and continuously mixed with a magnetic stirring bar (5,8,9). The bioassay was calibrated with authentic cADPR.

Chemicals. L. pictus was obtained from Marinus, Inc. (Long Beach, CA, USA). Fluo-3 was purchased from Molecular Probes; IP_3, oligomycin and antimycin were from Calbiochem. All other reagents were supplied from Sigma Co. (St. Louis, MO, USA). Stock solutions of all-*trans*-retinoic acid (RA), cortisol, estradiol and 1-dehydrotestosterone were dissolved in DMSO. 3,3′,5′-triiodothyronine (T_3) was dissolved in 6 mM NaOH.

Results were statistically evaluated using Student's t-test for paired comparisons or, when appropriate, by analysis of variance (ANOVA). Values are expressed as mean ± SEM and results were considered statistically different if P values were <0.05.

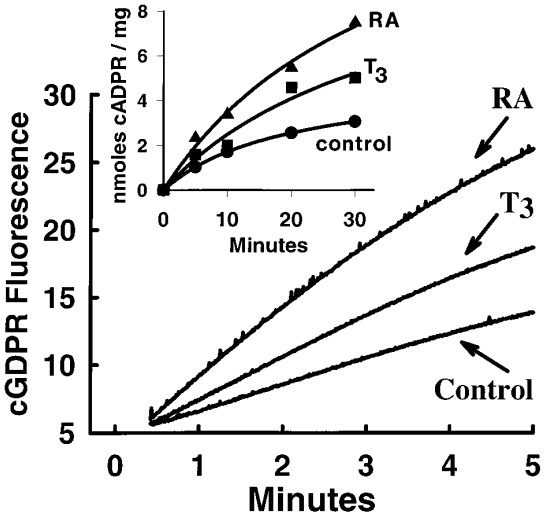

FIG. 1. ADPR-cyclase activity in VSMC treated with T_3 or RA. Cells were treated for 48 h with either vehicle (control), 1 μM T_3 or 1 μM RA. ADPR-cyclase was measured using NGD as a substrate. Picture depicts one representative experiment (n=6). *Inset:* cADPR synthesis from β-NAD^+ by membrane extracts of VSMC treated with either: vehicle (circles), 1 μM T_3 (squares) or 1 μM RA (triangles). Results represent means of 2 representative experiments.

RESULTS

In homogenates from VSMC, all ADPR-cyclase activity was found in the total membrane fraction obtained by sedimentation at 100,000 g for 90 min. (data not shown). Therefore, in subsequent experiments ADPR-cyclase was determined in total membrane fraction as defined in Methods.

Incubation of VSMC with RA or T_3 for 48 h resulted in a significant increase of ADPR-cyclase activity (Fig 1) when measured using either NAD or NGD as substrates. Reverse T_3 did not stimulate ADPR-cyclase activity (data not shown). The stimulatory effects of both RA and T_3 were concentration-dependent in the range of $10^{-9} - 10^{-6}$ M (Fig. 2). However, maximum stimulation by RA ($\cong$ +100%) was significantly higher than maximum stimulation by T_3 ($\cong$ +40%). The stimulatory effects of RA and T_3 upon ADPR-cyclase activity were additive (Fig. 3). Other hormones of the steroid superfamily, namely cortisol, β-estradiol and 1-dehydrotestosterone (DHT) showed no effect upon ADPR-cyclase in VSMC (Fig. 3).

Kinetic analysis revealed that stimulation of ADPR-cyclase activity by either RA or T_3 in VSMC was due

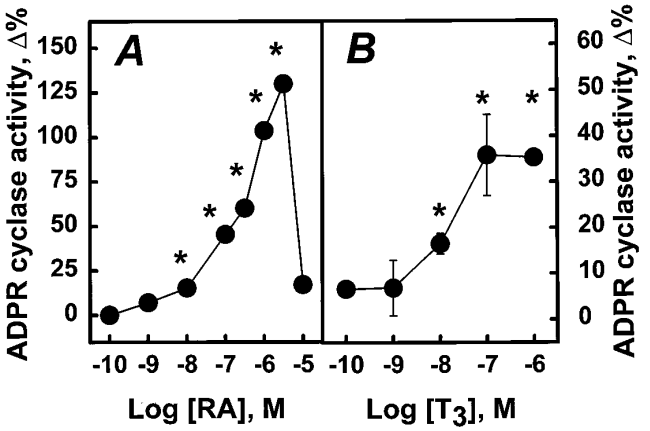

FIG. 2. RA and T_3 concentration-dependence of ADPR-cyclase activity. Cells were treated with different concentrations of RA or T_3 and ADPR-cyclase was measured and expressed as Δ% stimulation above control. (n=3, * indicates $P<0.05$ relative to control)

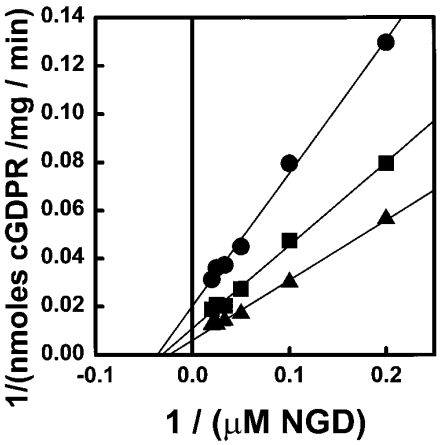

FIG. 4. Kinetic analysis of ADPR-cyclase activity in VSMC. Cells were treated either with vehicle (control) (circles), 1 μM T_3 (squares) or 1 μM RA (triangles). K_m and V_{max} values were determined by measuring the rate of cGDPR formation at different concentrations of NGD. Picture depicts one representative experiment of 4 independent experiments. Average K_m values were (in μM): 31±8, 31±8, 32±9 (control, T_3- and RA-treated cells, respectively). Average V_{max} values were (in nmoles cGDPR/mg/min): 83±23 (control); 109±28* (T_3); 159±38* (RA). (n=4, * indicates $p<0.05$ relative to control, paired t-test)

to an increase in V_{max} without changes in the apparent affinity for substrate (Km_{NGD}) (Fig. 4).

DISCUSSION

Results presented herein show that RA and T_3 increase ADPR-cyclase activity in VSMC grown in primary culture. In contrast, several other hormones of the steroid superfamily showed no effect, at least under present experimental conditions, indicating that the increase in ADPR-cyclase activity is not a general feature of the steroid superfamily of hormone action. However, it should be stressed out that an action of these hormones may still be detected under certain experimental conditions not employed herein as different cells may differ in their response in ADPR-cyclase to different hormones. For example, in our preceding studies, *in vivo* administration of β-estradiol to female rats stimulated ADPR-cyclase activity in myometrium (9) and in renal LLC-PK-1 cells ADPR-cyclase activity was stimulated by RA but not T_3. (4,5).

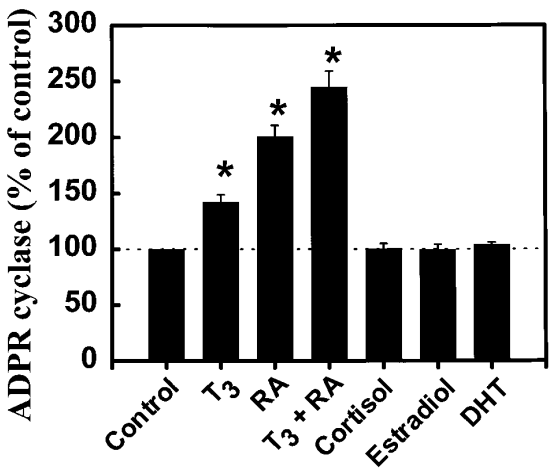

FIG. 3. Effect of T_3, RA, and steroids on ADPR-cyclase activity of VSMC. Cells were treated with either vehicle (control), 1 μM T_3, 1 μM RA, 1 μM of both T_3+RA, 1 μM cortisol, 10 nM estradiol or 10 nM 1-dehydrotestosterone (DHT). Results represent means of 3 experiments. * indicates statistically significant difference relative to control or other conditions where stimulation was observed (ANOVA).

Some genomic regulatory actions of RA and T_3 may overlap as expression of certain genes and corresponding protein products may be co-regulated by both hormones (16,17). Our finding that the stimulatory effects of RA and T_3 are additive (Fig. 3) suggests, in principle, that nuclear receptors for RA (RAR and RXR) and thyroid hormone (T_3R) can act at distinct hormone-responsive elements to stimulate ADPR-cyclase activity in VSMC.

Implications of present findings for biology and pathology of VSMC should be briefly considered. First, RA and T_3 may enhance cADPR-elicited Ca^{2+}-release in VSMC thereby promoting contractility. Indeed, RA has been shown to promote differentiation of VSMC towards a contractile phenotype (13). It is therefore possible that the effect of RA upon cADPR synthesis herein described might be part of a coordinated action of RA to induce differentiation to the contractile phenotype (13). Second, it should be recalled that cADPR was shown to trigger Ca^{2+}-release from the nuclear envelope into the nucleus (21), an event that may regulate Ca^{2+}-dependent gene expression (22,23).

There is very little information available about long-

term direct effects of T_3 upon VSMC contractility that are independent on β-adrenergic receptors. To our knowledge, this work is the first description of regulation of cADPR metabolism in eukaryotic cells by T_3, the biological relevance of which needs to be further investigated in future studies. It is possible that, akin to RA, thyroid hormone may increase cADPR synthesis to modulate contractility and specific gene expression.

In summary, we report that in VSMC synthesis of cADPR is upregulated by RA and T_3, two biological regulators of long-term control of cell proliferation, differentiation and expression of specific gene products. These findings are the basis of our hypothesis positing that in VSMC some of the biological responses to RA and T_3 are mediated through upregulation of cADPR generation and enhanced Ca^{2+}-release.

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