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Effect of adenosine and its metabolites on the hypothalamo-pituitary–adrenal axis

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Adenosine (ADO) plays a key role in maintaining the energy charge of the cell and has been shown in vivo to stimulate hypothalamo-pituitary–adrenocortical (HPA) activity. The question arises as to whether these effects are related exclusively to ADO and/or its metabolic product(s). Therefore, the present study was designed to test the in vivo effect of ADO and its phosphorylated or deaminated derivatives on plasma corticosterone concentration (PCC) in rats. ATP, ADP, AMP, ADO, inosine (INO), hypoxanthine (HYP), xanthine (XAN), and urate (URA) in solutions (40 μmol/100 g of metabolic body weight) were injected intraperitoneally, then 30 min later the animals were decapitated and the plasma samples were collected for corticosterone radioimmunoassay (RIA). Dose response curves for ADO and URA as well as a time course response for plasma URA and PCC following ADO administration were obtained. In addition, the effect of URA on the adrenocorticotrophic hormone (ACTH) secretion of AtT-20 pituitary cells in culture was determined. The results showed that not only ADO but the adenine nucleotides (AMP, ADP, ATP) and also the deaminated end-products (INO, XAN, URA) significantly increased PCC. HYP did not have any significant effect. The dose dependent effects of ADO and URA on PCC were significantly and highly correlated. URA stimulated ACTH secretion significantly in vitro in a dose-dependent manner, suggesting that ADO metabolites increase PCC via ACTH release. The possibility that ADO metabolites, principally URA, could be important signals for the HPA axis is discussed. (J. Nutr. Biochem. 6:334–339, 1995.)

Keywords: adenosine; inosine; xanthine; uric acid; ACTH; corticosterone

Introduction

Adenosine (ADO) and its metabolites have been shown to play an important role in the regulation of many physiological processes,[1–3] one of which is adrenocorticotrophic hormone (ACTH) corticosterone signaling.[4,5] Recently, Szabó et al.[6] reported that nutrients containing different caloric energy potentials elicit different plasma corticosterone concentrations (PCC) and they suggested that these effects may be mediated through ADO and/or its metabolites.

ADO occupies a central position in purine metabolism and has two primary metabolic pathways which are (1) reincorporation into the adenine nucleotide pool by phosphorylation or (2) deamination to inosine and its subsequent metabolites.[7] The two enzymes that compete for ADO are ADO kinase (phosphorylation) and ADO deaminase. At low concentrations, the ADO is phosphorylated to AMP, and at high concentrations it is deaminated to inosine (INO).[8,9] INO can be metabolized to hypoxanthine (HYP) and thus enter the purine salvage pathway, thereby recycling the HYP to INO-monophosphate and other nucleotides, or the HYP can be oxidized to xanthine (XAN) and uric acid (URA).

Although the in vitro and in vivo effects of ADO on the hypothalamo-pituitary-adrenal (HPA) axis are well documented,[5,6,10] it is unclear whether these effects are related exclusively to ADO and/or its metabolic product(s). Therefore, the present study was designed to test the effect of

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ADO and its metabolic phosphorylated or deaminated derivative(s) (adenosine triphosphate [ATP], adenosine diphosphate [ADP], adenosine monophosphate [AMP], XAN, HYP, INO, and URA) on PCC in rats in vivo, and to determine if URA, the end product of the ADO cascade, can directly stimulate the ACTH release of the pituitary cells in vitro.

Materials and methods

Animals

Male, 250–300 g, Wistar SPF rats (LATI, Gödöllö, Hungary) were housed individually in wire-bottom cages, and the room temperature was maintained at 23°C. The animals were fed rat chow (NRC-formulation, LATI, Gödöllö, Hungary) and provided drinking water ad libitum. Two weeks prior to the experiment, rats were handled every second day to acclimate them to human contact to minimize the stress response.

Experimental protocol in vivo

On the days of the experiments (8 to 11 a.m.), rats were weighed and the metabolic body weights were calculated (bwt^{75}). The animals were randomly divided into the following nine experimental groups (8 rats/group): (1) saline control (SAL, 0.15 mol/L NaCl), (2) ATP, (3) ADP, (4) AMP, (5) ADO, (6) INO, (7) HYP, (8) XAN, and (9) URA. ADO and its metabolites (the sodium salts of ATP, ADP, AMP, and URA) were dissolved in saline, and the pH of each solution was adjusted to 7.4 using 0.1 N HCl or NaOH. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO USA). The solutions were injected intraperitoneally (ip) based on 40 μmol/100 g of metabolic body weight (100 g of metabolic bwt = 464.5 g bwt); for ADO this is 23 mg/kg of bwt. If we assume that the water content of the body is ~65% and that ADO is evenly distributed in the extracellular fluid volume, then the extracellular ADO concentration would be ~123 μmol/L. The concentration of the administered ADO metabolites, including URA, were similar to ADO; however, the actual concentrations of URA may be higher due to endogenous URA concentration (e.g., [administered URA ≈ 123 μmol/L] + [endogenous URA ≈ 26 to 178 μmol/L as cited in the literature] = 149 to 301 μmol/L). While the actual URA concentrations were in the normal physiological range, the concentrations for ADO and its other metabolites were difficult to predict due to their rapid degradation to URA; the ADO concentration was based on that reported by Scaccianoce et al.[5]

Seventy-two rats (8/group) were used to determine the PCC dose response to ADO and URA (SAL: control; ADO: 2, 20, 40; and URA: 2, 5, 10, 20, 40 μmol/100 g of bwt^{75}). Additionally, a time course for PCC and URA, at 15, 30, 45, 60, 120, and 180 min, in the 40 μmol ADO/100 g of bwt^{75} was obtained (5 rats/group). Serum concentrations of URA were determined by an enzymatic colorimetric assay kit (Sigma Chemical Co., Cat. No. 685).

Szabó et al.[11] recently reported that maximal PCC could be attained ≈30 min following ip ADO administration in rats. Therefore, 30 min after injection of the test substances the rats were decapitated (except for the time course study). Decapitation occurred at 2-min staggered intervals between the various treatment groups in order to minimize any possible time effect due to circadian rhythm fluctuations between the groups and to randomize the time of sacrifice among the groups.

Experimental protocol in vitro

AtT-20 mouse anterior pituitary cell suspensions (CCL 89, Batch No. F-10522, 20th subculture; American Type Culture Collection, Rockville, MD USA) were maintained in Ham F-12 medium containing 2.5% fetal bovine serum, 15% horse serum, and 1% penicillin-streptomycin (GIBCO, Grand Island, NY USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C at a cell density of 10^5 to 10^6 cells/mL in T-75 flasks. Every second day, 5 mL of fresh medium was added to the cultures. The media was changed once per week, at which time the cells were subcultured (2×10^5 cells/mL) 20 hr prior to starting the experiment. The optimal conditions for the stimulation of ACTH secretion (cell density, incubation time, volume of the culture medium) were applied as described by Richardson.[12] For the experiments, 4 mL of cell suspension/sample, 8 samples/treatment were transferred into 50 mL polystyrene tubes, centrifuged, resuspended in the incubation medium (CO_2-equilibrated Ham-12 medium containing 0.1% bovine serum albumin [Sigma Chemical Co.], 10 mmol/L HEPES, pH 7.6 [Fisher Scientific, Cincinnati, OH USA], and 100 KIU/mL of aprotinin [protease inhibitor,[13] Boehringer Manheim, Indianapolis, IN USA]), preincubated for 60 min, then appropriate volumes of URA (12.5 mmol/L URA in 250 mmol/L of HEPES, pH 8.6) or isoproterenol stock solutions (ISO, 1 mmol/L of solution in 0.15 Dulbecco's phosphate buffer, pH 7.6, according to Shield[14]) were added and the samples were further incubated for 70 min. The final pH of the incubation medium was 7.6 at 37°C. Both URA and ISO were purchased from Sigma Chemical Co. Following the incubation in a humidified 5% CO_2-containing atmosphere at 37°C, the cells were centrifuged and the supernatant removed and stored at −70°C until assayed for ACTH. Protein concentrations were determined from the cell pellets using a Bio-Rad protein microassay with bovine gamma globulin as a standard (Bio-Rad Laboratories, Hercules, CA USA).

Hormonal assays

Blood samples were collected in heparinized test tubes, then the plasma was separated by centrifugation at 3,000 rpm for 10 min at 4°C and stored at −20°C prior to determination of corticosterone using radioimmunoassay (RIA). The intra-assay variation of the corticosterone RIA was 6.4%. The final dilution of the corticosterone antibody was 1:40,000. Cross-reactivity of the antiserum with other steroids was <0.11%, except with progesterone (2.3%) and desoxycorticosterone (1.5%). The calibration curve ranged from 0.01 pmol to 20 pmol/tube.

ACTH concentrations of the cell culture media were determined using a double antibody assay RIA kit (ICN Biomedicals, Inc., Costa Mesa, CA USA, Cat. No. 07-106101). The cross-reactivities of the antisera with ACTH was 100%, with α-MSH and β-MSH and β-endorphin were <0.1% while with β-lipotropin was <0.8%.

Statistical analysis

The data were analyzed by one-way ANOVA followed by a least square means (LSM) analysis or Scheffe's test to measure significant differences between treatment groups, using Abstat statistical analysis software (Anderson Bell Corp. Parker, CO USA).

Results

Effect of ADO and its derivatives on PCC

The data presented in *Figure 1* indicate clearly that not only ADO and its phosphorylated products but also its deami-

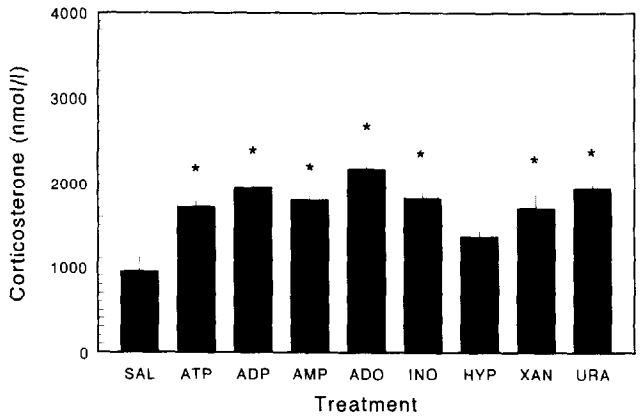

Figure 1 ADO and its metabolites increased the PCC. The compounds were administered ip, 40 μmol/100 g of bwt[75], 30 min prior to exsanguination. Each value is the mean ± SE, $n = 8$. *Significantly different from control (SAL) at $P < 0.05$. PCC = plasma corticosterone concentration, SAL = saline, ADO = adenosine, INO = inosine, HYP = hypoxanthine, XAN = xanthine, and URA = urate.

nated (INO) further oxidized products (XAN and URA) significantly increased PCC relative to the control (SAL) group. HYP did not have any significant effect.

ADO and URA both exhibited a significant and similar increase in PCC dose response (*Figure 2*). The correlations were highly significant at $P < 0.0001$, with a correlation coefficient of >0.98 for both ADO and URA. The maximal PCC response occurred ~30-45 min post-ADO administration (*Figure 3*). This PCC peak coincided with a concomitant increase in URA concentration. The correlation coefficient ($r = 0.965$) was significant at $P < 0.01$.

Effect of URA on the ACTH secretion of pituitary cells

URA at physiological concentrations, in a dose-dependent manner, significantly increased the ACTH release. The effect was not significantly different from that of ISO, a known ACTH-secretagog of AtT-20 cells (*Figure 4*).

Discussion

Effect of ADO and its metabolites on the HPA axis

All significant stress exerted on an organism initiates the neuroendocrine cascade of events resulting in glucocorticoid secretion from the adrenal cortex. Our experimental in vivo data suggest that in certain conditions, the stress response, i.e., increased PCC, is due to the effect of ADO and its metabolites. The effect of ADO and its analogs via purinoceptors on the cyclic AMP content of the pituitary and subsequent ACTH secretion appear to be well documented.[5,10,15] The extracellular nucleotides are rapidly broken down to ADO, INO, XAN, HYP, and URA,[16–19] thus ADO may not be the principal nucleotide signal for the HPA axis. Whether ADO has a direct effect in vivo on the

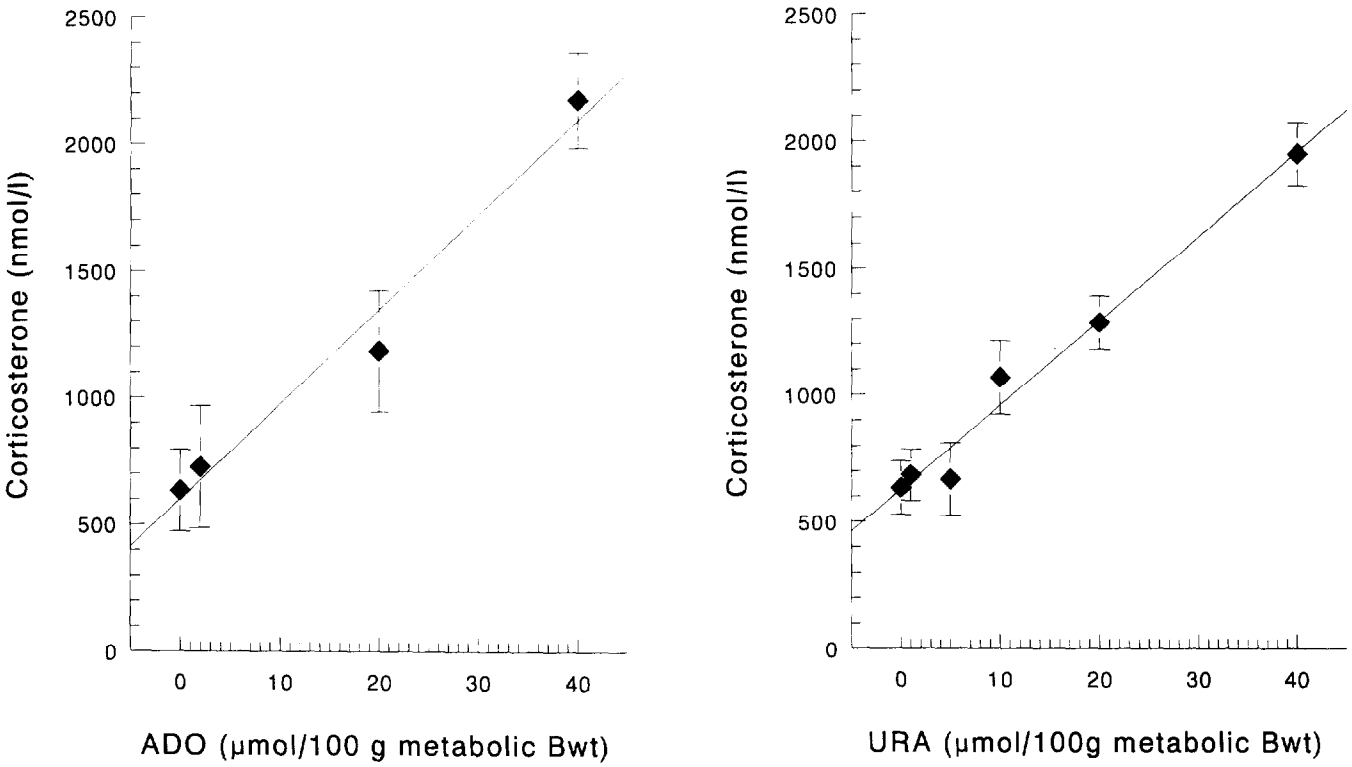

Figure 2 ADO and URA increased the PCC in a dose-dependent manner. Both compounds were administered ip 30 min prior to exsanguination. Each value is the mean ± SE ($n = 8$). Highly significant correlations, $P < 0.0001$ were observed for both ADO and URA, with nearly identical correlation coefficients, $r = 0.987$ and $r = 0.989$, respectively. ADO = adenosine, URA = urate, PCC = plasma corticosterone concentration.

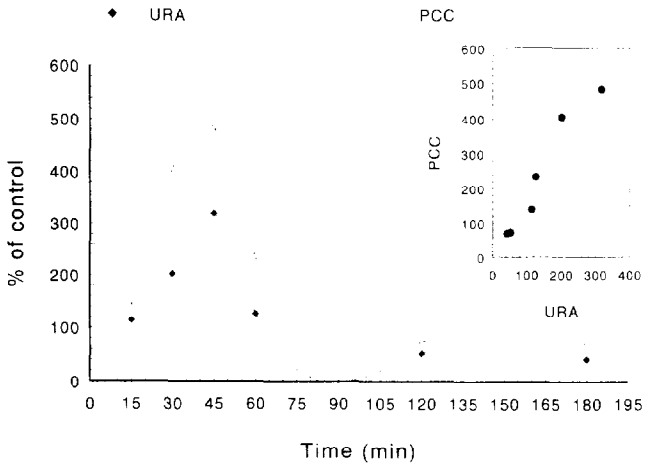

Figure 3 Following ADO ip injection (40 μmol/100 g of bwt[75]) the PCC and URA increased concomitantly, with a maximum at ~30 min. The data are expressed as percent of saline-administrated controls. Each value is the mean ± SE (n = 5). The correlation is significant ($P < 0.01$) with r = 0.965. ADO = adenosine, URA = urate.

pituitary purinoceptors or whether the effect is due mainly to its catabolic end product, URA, via an unknown mechanism, cannot be answered based on our present study. As shown in *Figure 3*, the URA peak coincides with the PCC peak which corroborates the finding of others that ADO is rapidly degraded to URA. We cannot explain, based on the current data, why HYP did not increase PCC; a time curve related to URA appearance following HYP administration may differ from that of ADO, INO, and XAN. The molecular mechanism of the effect of URA seems to differ from that of the ADO since URA apparently does not interact with brain ADO (A_1) receptors.[20] Our data clearly show that URA had a direct effect in vitro on the pituitary cells. It is unclear how the other catabolic products of ADO (INO, XAN, and HYP) may influence pituitary receptors. We suggest that the primary effect of ADO on HPA axis is most likely due to URA for the following reasons: (1) the PCC dose response curves obtained for URA and ADO were identical (*Figure 2*); (2) a significant correlation over time for URA and PCC was observed following ADO administration (*Figure 3*); (3) URA directly stimulated ACTH release in pituitary cells (*Figure 4*); (4) ADO's half life in the blood has been shown to be short, 0.6–1.5 sec[21]; (5) ADO deaminase added to anterior pituitary cells increased the ACTH secretion,[10] indicating that deaminated catabolites of ADO may increase the pituitary ACTH secretion; (6) in perfused isolated rat hearts, ADO accounted for only 4% of the purine released during energy expenditure, and in a reperfusion heart model the purine release was apparently all accounted for as URA[22,23]; (7) the ADO produced by myocytes is rapidly catabolized to URA by the endothelial cells prior to entering the systemic circulation[24]; (8) an apparent circadian rhythm for plasma URA has been shown to exist and handling stress altered the URA concentration in conscious cebus monkeys[25]; (9) our calculations based on the data of Filteau et al.[26] showed that URA accumulation in the hypothalamus of mice is directly correlated with PCC (r = 0.93).

It is apparent that ADO and its metabolites act at the pituitary level. ADO does not freely cross the blood–brain barrier[27] and its metabolites, which are lipid insoluble, behave in a similar manner. Indeed, the concentration of URA in the cerebrospinal fluid is one tenth the concentration found in plasma, reflecting the exclusionary function of the blood–brain barrier.[28] ADO does not appear to have a direct stimulatory effect on adrenocortical cells since in dexamethasone-treated or hypophysectomized rats in vivo neither ADO[5] nor ADO analogs[15,29,30] increase PCC. Since ADO and its nucleotides were administered ip and had to cross the capillary endothelial cell membrane prior to entering the systemic circulation, we postulate that the nucleotides and ADO, under aerobic conditions, are rapidly catabolized to URA and at least part of the observed PCC stimulatory effect of the ADO may be due to increasing plasma URA concentration. The results of our in vitro experiments support this hypothesis since URA applied in a physiological concentration increased the ACTH secretion of pituitary cells significantly and in a dose-dependent manner.

Metabolic stress: A proposed feedback control of the HPA axis by ADO/URA

We hypothesize that during stress the circulating end product of ADO metabolism, URA, may contribute to the regulation of the HPA axis. As shown in *Figure 5*, metabolic stress (caloric deprivation) may influence the phosphorylation potential (the cytosolic [ATP]/[ADP][Pi] ratio) or energy charge ([ATP] + ½[ADP])/[ATP] + [ADP] + [AMP]) of the cell. The activity of 5′-nucleotidase, the key enzymes responsible for degradation of AMP and IMP to ADO is controlled by the adenylate energy charge of cells.[31] The cellular purine release is directly related to the rate of energy consumption and inversely related to the rate of energy production in the isolated heart.[23] Since the primary end product of purine metabolism is URA, it is possible that the circulating concentration may influence the

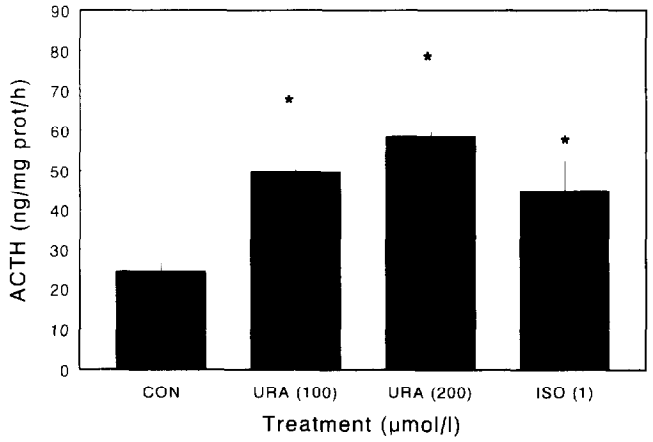

Figure 4 The uric acid (URA) increased the ACTH content of the cell culture media of AtT-20 mouse pituitary cells in a dose-dependent manner, similar to that of isoproterenol (ISO). Each value is the mean ± SD, n = 8. *Significantly different from unstimulated control (CON) at $P < 0.01$. CON = control (30 mmol/L of HEPES buffer, pH 7.6), URA (100) = URA 100 μmol/L, URA (200) = URA 200 μmol/l, ISO (1) = isoproterenol 1 μmol/L.

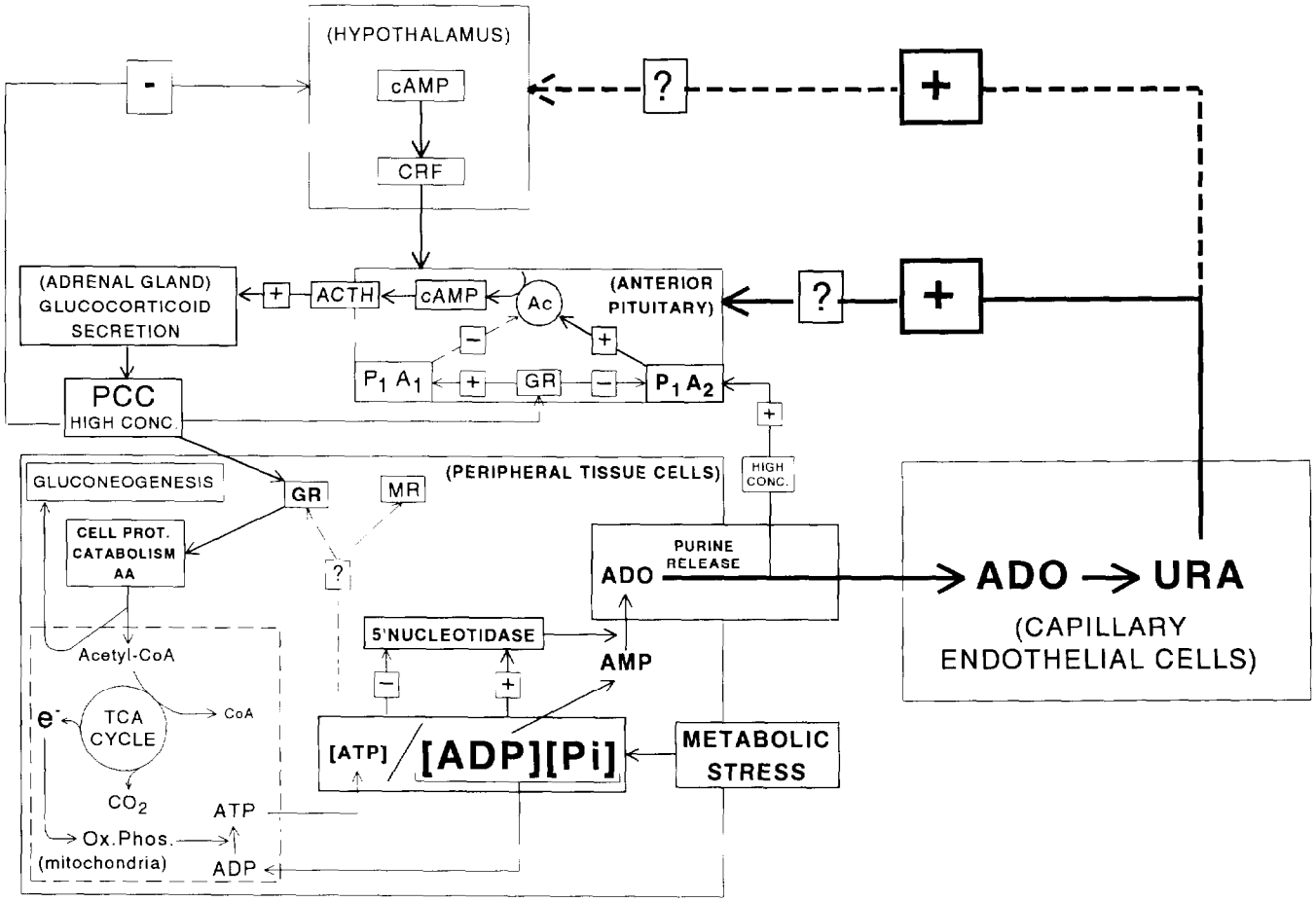

Figure 5 Schematic diagram of a proposed feedback control mechanism for stimulation of the hypothalamo-pituitary-adrenal axis. The metabolic stress influences the phosphorylation potential (the cytosolic [ATP]/[ADP][Pi] ratio) or energy charge ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP]) of the cell. The resulting cellular purine release—directly related to the rate of energy consumption and inversely related to the rate of energy production, via ADO and/or URA—could serve as a signal to the pituitary cells and stimulate the ACTH release. In turn, the rate of ACTH release influences the plasma glucocorticoid concentration, thus the cellular catabolism according to the cells' physiological requirements. CRF = corticotropin-releasing factor. Ac = adenylate cyclase, cAMP = cyclic AMP, P_1A_1 and P_1A_2 = purinoceptors, ADO = adenosine, URA = uric acid, GR = type II or glucocorticoid receptors, MR = type I or mineralocorticoid receptors, PCC = plasma corticosterone concentration, and Ox. Phos. = oxidative phosphorylation.

HPA axis and PCC by an as yet unknown mechanism(s). Caloric deprivation may activate the HPA axis as recently reported by Hanson et al.[32] The concentrations of URA, used for establishing the dose response curve for PCC, are within the normal physiological range observed for rats. High concentrations of ADO, however, may directly influence the pituitary A_2 receptors and increase cAMP concentration and ACTH secretion[5,10,33] (*Figure 5*). Since the A_2 agonist concentration of ADO in in vitro conditions is in the μmol/L range,[5] it is unlikely that the ADO concentrations used in our experiment reached an effective plasma level to facilitate A_2 receptor binding because of its rapid degradation. However, we cannot exclude the effect of ADO because its plasma level was not measured. The normal circulating level of ADO, 79 nmol/L in the rat,[34] is below the concentration needed for A_2 binding and even under hypoxic stress is only elevated to 190 nmol/L[34]; this concentration is still far from the effective pituitary A_2 micromolar binding affinity for ADO, again suggesting that the most likely agonist for the HPA axis is URA.

In summary, ADO and its phosphorylated/deaminated end products stimulated the HPA axis in vivo in rats. Based on these data and our finding that URA stimulated ACTH production in the pituitary cells, we postulate that under aerobic circumstances URA may act as the primary signal for the HPA axis.

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