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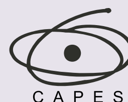

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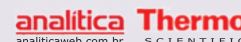

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Arginine induces GH gene expression by activating NOS/NO signaling in rat isolated hemi-pituitaries

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Abstract

The amino acid arginine (Arg) is a recognized secretagogue of growth hormone (GH), and has been shown to induce GH gene expression. Arg is the natural precursor of nitric oxide (NO), which is known to mediate many of the effects of Arg, such as GH secretion. Arg was also shown to increase calcium influx in pituitary cells, which might contribute to its effects on GH secretion. Although the mechanisms involved in the effects of Arg on GH secretion are well established, little is known about them regarding the control of GH gene expression. We investigated whether the NO pathway and/or calcium are involved in the effects of Arg on GH gene expression in rat isolated pituitaries. To this end, pituitaries from approximately 170 male Wistar rats (~250 g) were removed, divided into two halves, pooled (three hemi-pituitaries) and incubated or not with Arg, as well as with different pharmacological agents. Arg (71 mM), the NO donor sodium nitroprusside (SNP, 1 and 0.1 mM) and a cyclic guanosine monophosphate (cGMP) analogue (8-Br-cGMP, 1 mM) increased GH mRNA expression 60 min later. The NO acceptor hemoglobin (0.3 µM) blunted the effect of SNP, and the combined treatment with Arg and L-NAME (an NO synthase (NOS) inhibitor, 55 mM) abolished the stimulatory effect of Arg on GH gene expression. The calcium channel inhibitor nifedipine (3 µM) also abolished Arg-induced GH gene expression. The present study shows that Arg directly induces GH gene expression in hemi-pituitaries isolated from rats, excluding interference from somatostatinergic neurons, which are supposed to be inhibited by Arg. Moreover, the data demonstrate that the NOS/NO signaling pathway and calcium mediate the Arg effects on GH gene expression.

Key words: Arginine; GH mRNA expression; Nitric oxide; Calcium (Ca^{2+}); Cyclic guanosine monophosphate

Introduction

Amino acids are essential molecules for protein synthesis, being fundamental for the growth and development of all life forms (1). In particular, the amino acid L-arginine (Arg) plays a crucial role in nutrition and physiology, as its dietary restriction leads to growth delay. This event does not seem to be associated with the primary function of amino acids as building blocks for proteins, since the restriction of some other amino acids does not interfere with this process, a fact that reinforces the importance of Arg for growth (2).

Indeed, Arg was shown to stimulate the secretion of hormones that are involved in growth and metabolism, such as insulin and growth hormone (GH) (3-8). However, the exact mechanism by which Arg stimulates the secretion of both hormones is not completely known.

It has been pointed out that Arg suppresses the release of somatostatin (4,7,8), which is known to exert an inhibitory effect on insulin and GH secretion. Moreover, Arg treatment increased calcium influx in pancreatic beta and pituitary cells in culture, suggesting a stimulatory effect on the secretion of insulin and GH (9-11).

It has also been shown that Arg is the natural precursor of nitric oxide (NO), which is involved in several physiological events (12-14), and whose synthesis is mediated by NO synthase (NOS) activation. The effects of NO require the enhancement of the levels of nucleotide cyclic guanosine monophosphate (cGMP), a second messenger that activates protein kinase G (PKG), which promotes phosphorylation of proteins responsible for triggering the

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effects of NO (15).

The enzyme that produces NO is expressed in several tissues, including pituitary cells (16,17). Therefore, a connection between the actions of Arg on the somatotrophs and NO production could exist. In fact, potent stimulators of GH secretion, such as growth hormone-releasing hormone (GHRH) and ghrelin, also exert their effects on the somatotrophs by the induction of NOS activity and, consequently, NO production (18,19).

Although several studies have indicated that Arg induces GH secretion, less emphasis has been given to the effects of Arg on GH gene expression.

Studies carried out in our laboratory indicated a role for Arg in GH gene expression, which was characterized by the increase of GH transcript content in rat pituitaries and GH3 cells 1 h after Arg treatment (20). However, the mechanisms involved in this effect are still unknown. The present study attempted to investigate this issue by evaluating the involvement of NO signaling pathways in the Arg-induced GH gene expression, in isolated rat hemi-pituitaries.

Material and Methods

Materials

Arginine was kindly provided by Laboratórios Baldacci (Brazil). Ketamine and xylazine were purchased from Davol Comércio e Representações Ltda. (Brazil). Modified Eagle's medium (MEM), phosphate-buffered saline (PBS), N_ω-nitro-L-arginine methyl ester (L-NAME), nifedipine, sodium nitroprusside (SNP), hemoglobin, and 8-bromoguanosine-3':5'-cyclic monophosphate (8-Br-cGMP) were purchased from Sigma Chemical Co. (USA). Phenol, formamide, guanidine isothiocyanate, sodium N-lauryl sarcosine, diethyl pyrocarbonate (DEPC), salmon sperm DNA, DNase, agarose, N-morpholino-propanesulfonic acid buffer (MOPS), sodium dodecyl sulfate, random primer labeling system kit, and ethidium bromide were purchased from Invitrogen Life Technology (USA). Films for X-ray, developer and replenisher were purchased from IBF (Indústria Brasileira de Filmes, Brazil). The MAXIscript^{TM} T3/T7 kit - *In vitro* RNA Transcription kit was purchased from Ambion (USA). Bovine serum albumin (BSA) and ß-mercaptoethanol were purchased from Life Technologies, Inc. (USA). High-quality nylon membranes were purchased from Bio-Rad Laboratórios Brasil Ltda. (Brazil). [^{32}P]-CTP and [^{32}P]-UTP were purchased from PerkinElmer do Brasil Ltda. (Brazil). All other reagents were purchased from Labsynth (Brazil).

Animals and treatments

Male Wistar rats weighing approximately 250 g obtained from our own breeding colony were used in the experiments. The animals were housed under conditions of constant temperature (23 ± 1°C) on a 12 h-light, 12-h dark (lights on at 7:00 am) schedule. Standard rat chow and tap water were provided *ad libitum*. The pituitaries from these animals were used for the studies, as described below. All experiments were performed in the morning between 9:00 and 11:00 am and repeated three times at the same time of day. The experimental protocols are in accordance with the ethical principles in animal research adopted by the Brazilian College of Animal Experimentation (COBEA) and were approved by the Ethics Committee of Instituto de Ciências Biomédicas, Universidade de São Paulo, for Animal Research (CEEA). Before the experiments, the animals were weighed, anesthetized, and killed by decapitation.

The experimental protocol used was adapted from Bianco et al. (21) and Hefco et al. (22), and the modifications are described below. The pituitaries were excised by an aseptic technique, washed once in 1X PBS, and sectioned longitudinally in two halves. Each half was placed on 2 different plates, and 2 pools of 3 hemi-pituitaries each were prepared. One pool was used for the control group and the other for the experimental groups. Subsequently, hemi-pituitaries were pre-incubated in 1 mL MEM under controlled conditions (95% O_2 and 5% CO_2 at 37°C).

After 30 min, the medium was removed and replaced with MEM containing saline or different compounds, such as 71 mM Arg, 55 mM L-NAME, 3 µM nifedipine, 10 nM, 1 µM, 0.1 mM, and 1 mM SNP, 0.3 µM hemoglobin, and 0.1 and 1 mM 8-Br-cGMP for 60 min. At the end of incubation, the medium was removed once again and the hemi-pituitaries were subjected to total RNA extraction. The dose of Arg used in the experiments was chosen from the dose-response (7.1, 71, 710 mM) and time-course study (10, 20, 30, 60, and 120 min). Each experiment included 3 rats per group and was repeated three times.

Total RNA extraction and Northern blotting analysis

Total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform extraction method (23) and quantified by absorbance at 260 nm. Total RNA samples were denatured with formaldehyde-formamide and submitted to electrophoresis on 1% agarose gels containing 2.2 M formaldehyde in 1X MOPS and blotted to a nylon membrane by neutral capillary transfer. The membrane was dried at 80°C for 1 h in a vacuum oven and pre-hybridized in 50% formamide hybridization solution and 100 g/mL denatured salmon sperm DNA at 42°C for 4 h. Subsequently, the membrane was incubated with a [^{32}P]-labeled rat GH cDNA by random priming for 16 h at 42°C. The membrane was washed under high-stringency conditions, subjected to autoradiography and quantified by phosphor imaging using the ImageQuant software (Molecular Dynamics, USA). All membranes were stripped and re-hybridized with a [^{32}P]-labeled RNA probe specific for 18S ribosomal RNA (18S c-rRNA), synthesized by *in vitro* transcription, to correct the variability in RNA loading. The results are reported as means ± SEM GH mRNA/18S rRNA ratio.

Statistical analysis

Data are reported as means ± SEM and were subjected to analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. The Student *t*-test was applied when appropriate. The level of significance was established at 5% (P < 0.05).

Results

Effect of different doses of arginine on GH mRNA expression

Figure 1 shows the GH mRNA response of hemi-pituitaries to 7.1, 71, 710 mM Arg added to the culture medium for 60 min. It can be seen that 7.1 mM Arg did not change GH mRNA content compared to control. However, a 10 times higher dose of Arg (71 mM) led to an increase in GH transcript level. In contrast, a 100 times higher dose of Arg (710 mM) did not alter GH mRNA content, which remained similar to control. Therefore, the subsequent experiments in which the effect of Arg was tested were performed using the dose of 71 mM.

Time-course study of GH gene expression in response to arginine

Considering that GH mRNA content was shown to be increased 60 min after the hemi-pituitaries were incubated with 71 mM Arg, in the present study we evaluated the effect of this dose of Arg on this parameter during shorter (10, 20, and 30 min) periods of time and periods longer than 60 min (120 min). Figure 2 shows these data, which demonstrate that GH mRNA content was not altered 10, 20, and 30 min after Arg treatment. However, when the time of incubation was extended to 120 min, a significant increase of GH mRNA content was observed. This result indicates that, from 60 min to at least 120 min of Arg incubation, the GH mRNA content remained increased; hence, in the subsequent experiments, we decided to use the shorter period of time during which this dose of Arg led to the increase of GH mRNA.

Arginine induces GH mRNA expression through NO generation

To evaluate the involvement of NO in the Arg-induced GH mRNA expression, we added an L-NAME, a known NOS inhibitor, to the culture medium for the hemi-pituitaries in the presence or absence of Arg. These data are presented in Figure 3, which shows that L-NAME by itself did not alter GH gene expression; however, when associated with Arg, this compound prevented the increase of GH transcript content induced by the amino acid.

NO increases GH mRNA expression

To determine whether NO increases GH mRNA expression, hemi-pituitaries were incubated with SNP, a well-known NO donor, at the following concentrations: 1 mM, 0.1 mM, 1 µM, and 10 nM. Figure 4A illustrates this experiment, and shows that SNP was able to increase GH mRNA expression at the higher concentrations used (1 and 0.1 mM). However, when hemoglobin, which is an NO acceptor, was added to the culture medium concomitantly with SNP, the effect of the latter in increasing GH mRNA content was abrogated (Figure 4B).

Considering that the actions of NO are mediated by cGMP, a stable analogue of 8-Br-cGMP (1 mM) was added to the hemi-pituitary culture medium to evaluate GH mRNA expression. As shown in Figure 4C, 8-Br-cGMP led to an increase of GH gene expression, an effect that was com-

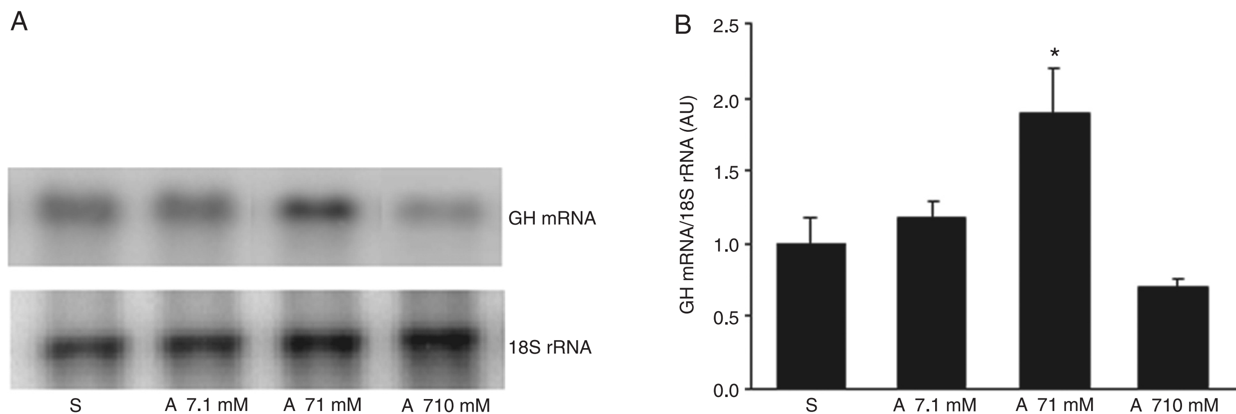

Figure 1. Dose-response study of the effect of arginine on growth hormone (GH) mRNA expression. *A*, Typical autoradiograms of the GH mRNA and 18S abundance obtained in one experiment of a total of three. *B*, Quantitative representation of hybridization of GH and 18S rRNA transcripts obtained in all experiments. Hemi-pituitaries were incubated for 1 h with saline (S) or arginine (A) at the concentrations of 7.1, 71, and 710 mM. The values obtained from blot densitometry were normalized by 18S rRNA. Data are reported as means ± SEM in arbitrary units (AU). *P < 0.01 *vs* all other groups (one-way ANOVA followed by the Student-Newman-Keuls test).

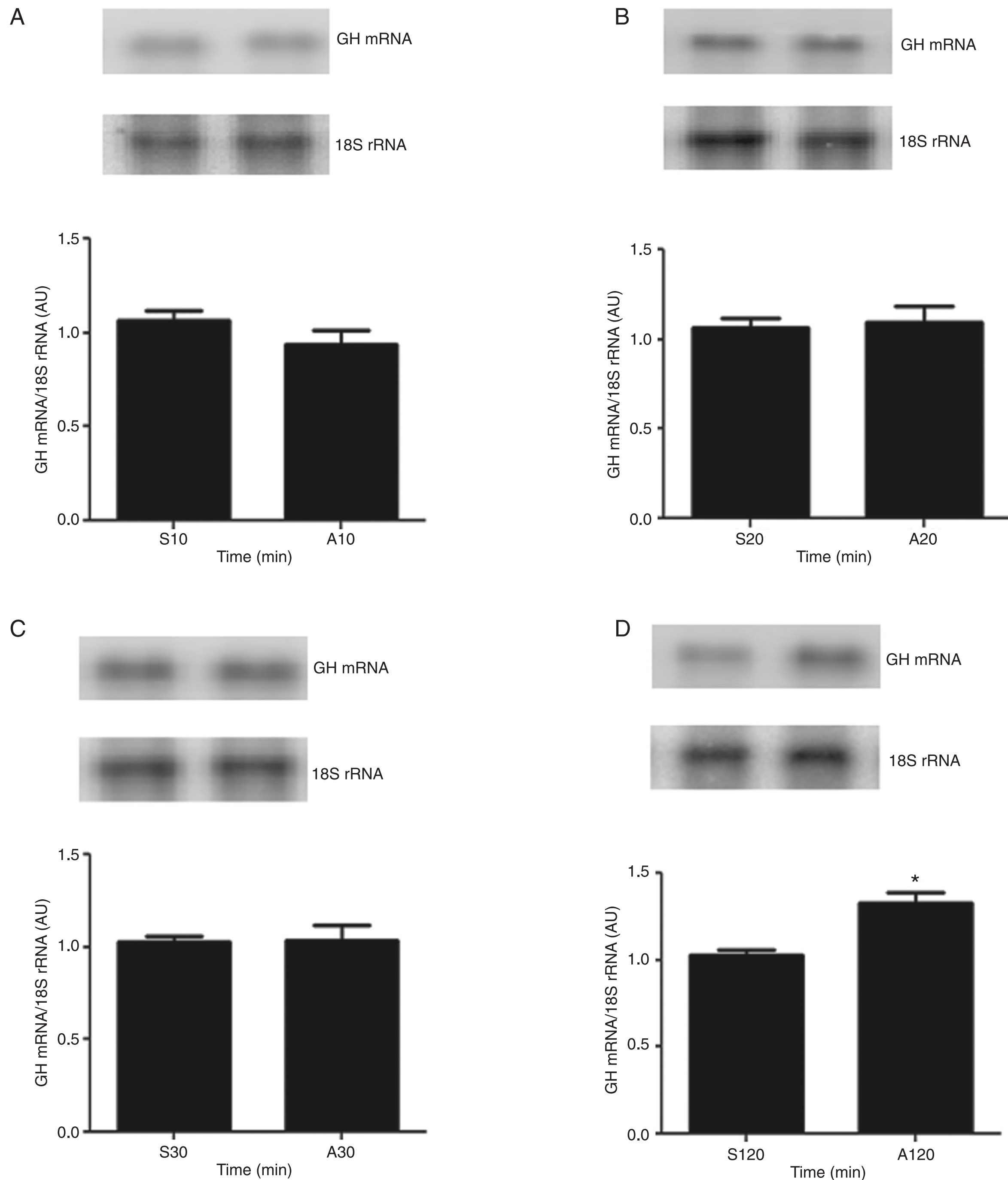

Figure 2. Time-course study of the effect of arginine on growth hormone (GH) mRNA expression. *Panels A*, *B*, *C*, and *D* present at the top typical autoradiograms of the GH mRNA and 18S abundance obtained in one experiment of a total of three for hemi-pituitaries treated with saline (S) or 71 mM arginine (A) for 10, 20, 30, and 120 min, respectively. The quantitative representation of hybridization of GH and 18S rRNA transcripts obtained in all experiments is shown at the bottom of each panel. Data are reported as means ± SEM in arbitrary units (AU). *P < 0.05 *vs* saline (Student *t*-test).

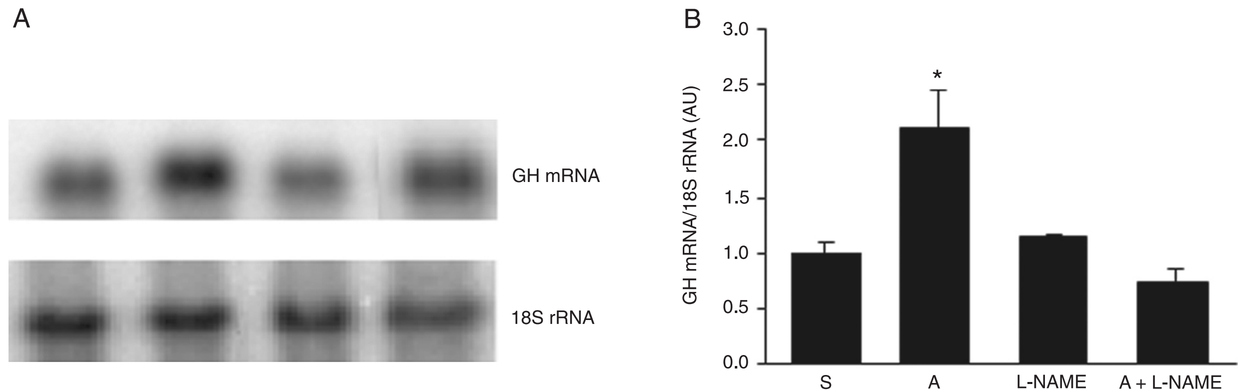

Figure 3. Effect of L-NAME plus arginine administration on growth hormone (GH) mRNA expression. *A*, Typical autoradiograms of the GH mRNA and 18S abundance obtained in one experiment of a total of three. *B*, Quantitative representation of hybridization of GH and 18S rRNA transcripts obtained in all experiments. Hemi-pituitaries were incubated for 1 h with saline (S) or 71 mM arginine (A) in the presence or absence of 55 mM L-NAME (L-NAME and A+L-NAME). The values obtained from blot densitometry were normalized by 18S rRNA. Data are reported as means ± SEM in arbitrary units (AU). *P < 0.001 *vs* all other groups (one-way ANOVA followed by the Student-Newman-Keuls test).

Figure 4. Effect of nitric oxide on growth hormone (GH) gene expression. *Panel A*, Hemi-pituitaries incubated for 1 h with saline (S) or sodium nitroprusside (SNP) at the following concentrations: 10 nM, 1 µM, 0.1 mM, and 1 mM. *Panel B,* Hemi-pituitaries were incubated for 1 h with S or 0.1 mM SNP in the presence or absence of 0.3 µM hemoglobin (Hb and SNP+Hb). *Panel C*, Hemi-pituitaries incubated for 1 h with S or 8-Br-cGMP at the concentrations of 0.1 and 1 mM. Typical autoradiograms of the GH mRNA and 18S abundance obtained in one experiment of a total of three are shown at the top of each panel, and the quantitative representation of hybridization of GH and 18S rRNA transcripts obtained in all experiments is shown at the bottom of each panel. The values obtained from blot densitometry were normalized by 18S rRNA. Data are reported as means ± SEM in arbitrary units (AU). *A*, *P < 0.05 *vs* S, 0.1 mM SNP and 1 mM SNP; *B*, *P < 0.05 *vs* all other groups; *C*, *P < 0.05 *vs* all other groups (one-way ANOVA followed by the Student-Newman-Keuls test).

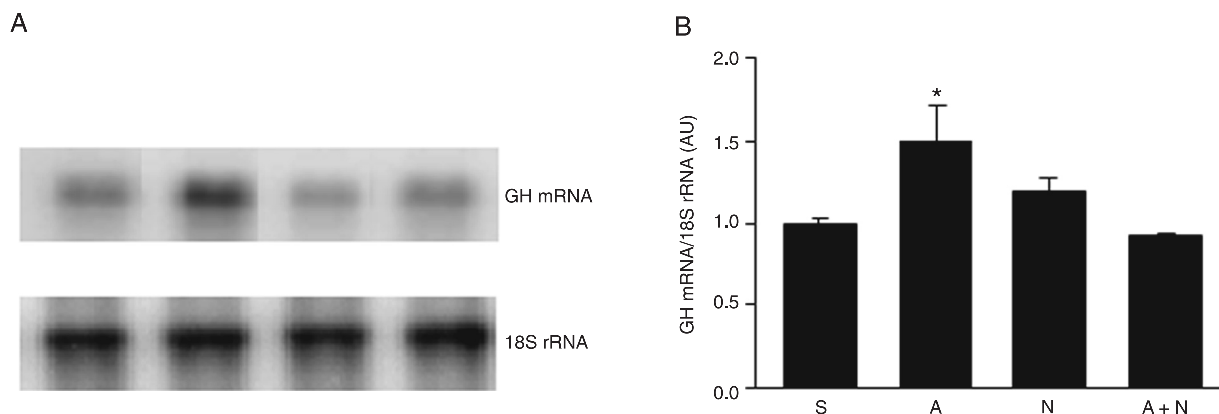

Figure 5. Effect of nifedipine plus arginine on GH gene expression. *A*, Typical autoradiograms of the GH mRNA and 18S abundance obtained in one experiment of a total of three. *B*, Quantitative representation of hybridization of GH and 18S rRNA transcripts obtained in all experiments. Hemi-pituitaries were incubated for 1 h with saline (S) or 71 mM arginine (A) in the presence or absence of 3 µM nifedipine (N and A+N). The values obtained from blot densitometry were normalized by 18S rRNA. Data are reported as means ± SEM in arbitrary units (AU). *$P < 0.05$ *vs* all other groups (one-way ANOVA followed by the Student-Newman-Keuls test).

parable to those observed after Arg or SNP treatment.

Calcium is involved in arginine-induced GH mRNA expression

To evaluate whether Ca^{2+} is required for the effects of Arg on GH mRNA expression, an L-type Ca^{2+} channel blocker, nifedipine (3 µM), was added to the hemi-pituitary culture medium in the presence or absence of Arg. Figure 5 shows that the stimulatory effect of Arg on GH gene expression was completely abolished by nifedipine, whereas nifedipine alone had no effect on GH gene expression.

Discussion

Arginine is a conditionally essential amino acid that was shown to stimulate GH gene expression in *in vivo* and *in vitro* studies (20) by mechanisms that were poorly understood. In the present study, we used hemi-pituitaries of rats incubated in the presence of 71 mM Arg for 60 min, in an attempt to determine the mechanism that underlies this effect. This dose and time of incubation period were selected after detecting that Arg is able to increase GH mRNA content in a dose- and time-independent manner. For this reason, we chose only one dose of Arg and the shortest time tested when its effect was observed. In fact, the use of a 10 times higher dose of Arg (710 mM) led to a decrease in GH transcript amount compared to the results obtained with 71 mM Arg. It is known that high concentrations of arginine may generate high concentrations of NO, and the excess of NO and, as described (24), its derivatives might be associated with the inhibition of mitochondrial respiration, a mechanism that could affect GH gene expression.

Arg is the natural precursor of NO, whose generation depends on the activity of NOS, an enzyme expressed in many tissues, including the pituitary cells (25), that catalyzes the oxidation of Arg to citrulline and NO (26). NO acts as an intracellular and intercellular mediator in many physiological processes (15), and it was shown to be implicated in the regulation of the activity of the hypothalamus-pituitary axis. NO can also regulate the expression of many genes, like the CYP4A in liver and kidney (27) and glial fibrillary acidic protein (GFAP) in astrocytes (28), and an involvement of NOS and NO in the enhancement of GH gene expression by Arg was also pointed in the current study.

Indeed, the inhibition of NOS activity by the addition of L-NAME to the hemi-pituitaries incubated in the presence of Arg prevented its stimulatory effect on GH gene expression, indicating that the products generated by NOS were mediating the increase of the GH mRNA content. As already mentioned, the main product generated from NOS activity is NO, which exerts important actions on the pituitaries, such as the induction of GH secretion (18,19). Therefore, it is possible that NO may enhance GH gene expression, since the effects of Arg increasing GH mRNA content were abolished by L-NAME, as pointed out here.

The addition of SNP, a potent NO donor, to the hemi-pituitary incubation medium also increased GH gene expression, providing evidence of a direct effect of NO, which is supposed to be the main metabolite involved in the Arg-induced GH gene expression. Moreover, the concomitant addition of SNP and hemoglobin, which is an NO acceptor, abrogated the increase of GH mRNA induced by SNP alone, reinforcing the role of NO in the control of somatotroph GH gene expression.

Our data agree with reports indicating a stimulatory effect of GHRH or ghrelin on GH secretion, which relies on

the activation of NOS and NO production (18,19). Since GHRH is the most important stimulator of GH synthesis and secretion, and NO is involved in the GHRH signaling pathway, our findings strongly indicate that NO might control the function of somatotrophs, regulating the GH gene expression as well. Evidence of a role of NO in the control of GH gene expression was also suggested by studies showing that leptin regulates GH gene expression and also stimulates NO release in pig pituitary cells (25).

To exert its effects, NO needs to recruit and activate guanylate cyclase, an enzyme that converts guanosine triphosphate (GTP) to cGMP. cGMP is a second messenger that binds to PKG, leading to conformational changes in its structure, and, as a consequence, to its activation. This enzyme is responsible for the actions of NO on the organic system (29); hence, the effects of NO on cells are associated with increased intracellular cGMP content (30).

This rationale was used in the experiment in which hemi-pituitaries were incubated with 8-Br-cGMP, a cGMP analogue, which may mimic the actions of NO (18). 8-Br-cGMP definitely led to an increase in GH mRNA content, indicating that the components of the NO signaling pathway are indeed involved in this mechanism.

In the present study, calcium ion was also shown to be involved in the effects of Arg on somatotrophs, since the inhibition of its influx into pituitaries by nifedipine prevented the stimulatory effect of Arg on GH gene expression. Even though the precise mechanism by which calcium is implicated in this response is unknown, it is recognized that calcium is an inductor of NOS activity, consequently leading to an increase in NO (31). However, other mechanisms could be activated by calcium. It is known that calcium influx through L-type voltage-gated calcium channels is required for the expression of specific genes. Indeed, the increase of intracellular Ca^{2+} concentration was shown to lead to the activation of cyclic AMP-responsive element binding protein (CREB), and subsequently to the induction of somatostatin gene expression (32).

In fact, it is well known that GHRH stimulates GH gene expression through cAMP-mediated protein kinase A, which phosphorylates and activates CREB (33). As mentioned above, one of the best characterized signals for CREB phosphorylation and activation is an increase in intracellular Ca^{2+} concentrations (32,34). Activated CREB is known to enhance Pit-1 gene transcription, and, as a consequence, to enhance GH gene expression (35). Definitely, the presence of calcium is essential for the action of Arg on somatotrophs, such as GH release and GH gene expression.

Taken together, the present data indicate a clear and direct effect of the amino acid Arg on the increase of GH mRNA content by means of NO generation, because L-NAME was able to prevent the increase of GH gene expression. NO is known to increase cGMP, and this study also showed that NO donors, as well as cGMP, which is supposed to mediate the effects of NO, increase GH gene expression. Calcium entry appeared to be necessary for the induction of GH gene expression by Arg. Hence, in parallel to its recognized effect on GH release, Arg also increases GH gene expression by activating the NOS/NO signaling pathway. These data increase the body of evidence showing that dietary constituents can modulate events that regulate gene expression, therefore affecting specific aspects of cellular function.

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