Involvement of Cyclic Guanosine 3′,5′-Monophosphate in Nitric Oxide-Induced Glucagon Secretion From Pancreatic Alpha Cells

Toshiaki Mori, Yoshio Murakami, Kunio Koshimura, Kazuyuki Hamaguchi, and Yuzuru Kato

It has been reported that nitric oxide (NO) is a positive modulator of glucagon release. The involvement of cyclic guanosine 3′,5′-monophosphate (cGMP) in NO-induced glucagon secretion and the possible role of NO in glucagon release induced by L-arginine were investigated in mouse clonal α-cell line clone 6 (αTC6) cells, which predominantly secrete glucagon. NOC12, an NO donor, elicited an increase in glucagon release from αTc6 cells in perifusion and static incubation. An inhibitor of cGMP-dependent protein kinase inhibited NOC12-induced glucagon release. NOC12 (1 mmol/L) also increased the cellular level of cGMP. In addition, a permeable cGMP agonist increased glucagon release. L-arginine (15 mmol/L) increased perifusate concentrations of glucagon and nitrite in αTc6 cells, which were inhibited by N^G-nitro-L-arginine methyl ester. NO synthase (NOS) activity was shown in αTc6 cells by L-citrulline formation assay. Our present findings suggest that NO plays a stimulating role in glucagon release from the α cells, and that a cGMP-dependent pathway is involved in NO action. These findings also provide further evidence that L-arginine might play a stimulating role in regulating glucagon secretion, at least partly, through generation of NO in the islets.

NITRIC OXIDE (NO) is a biomediator with widespread biologic actions in many tissues.[1] The role of intracellular cyclic guanosine 3′,5′-monophosphate (cGMP) in NO action has been implicated.[2,3] NO is the product of the oxidation of the guanidine nitrogen of L-arginine by NO synthase (NOS). Further, the occurrence of NOS activity has been shown in the pancreatic islet.[4-7]

There is a growing body of evidence to suggest that NO might play roles in islet hormone secretion. Although the initial reports described NO as enhancing insulin release in clonal β-cell lines,[4,8] subsequent studies have suggested that NO is a negative modulator of insulin release in the normal islets of Langerhans.[7,9-12] Reportedly, it was plausible that the suppressive effect of NO on insulin secretion was being mediated through the formation of S-nitrosothiols[13] rather than the elevation of intracellular cGMP.[9,14] It is now conceivable that NO is a positive modulator of glucagon release.[7,11,12,15,16] The mechanisms involved in NO-induced glucagon secretion, however, remain to be elucidated. The possible role of insulin, a physiologic inhibitor of glucagon secretion,[17,18] should be considered whenever experiments are performed that use pancreatic isles containing distinct cell populations.

In the present study, the involvement of cGMP in NO-induced glucagon secretion was investigated in mouse clonal α-cell line, αTC6 cells. Because αTC6 cells predominantly secrete glucagon, but not insulin,[19] we could exclude any effect of insulin on glucagon secretion. The possible role of NO in L-arginine-induced glucagon release was also studied in these cells.

MATERIALS AND METHODS

Reagents

3-Ethyl-3-(ethylaminoethyl)-1-hydroxy-2-oxo-1-triazene (NOC12) was from Dojindo Laboratories, Kumamoto, Japan; L-arginine · HCl, N^G-nitro-L-arginine methyl ester (L-NAME) and isobutylmethylxanthine (IBMX) were from Nacalai Chemical, Kyoto, Japan; 8-bromoguanosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cGMP) was from Calbiochem-Novabiochem, San Diego, CA; guanosine-3′,5′-cyclic monophosphorothioate, Rp-isomer (Rp-cGMPS) was from Biolog Life Science Institute, Bremen, Germany. All other chemicals were of the purest grade available from regular commercial sources.

Cells

Clonal mouse pancreatic α-cell line, αTC clone 6, (αTC6 cells) were used. Cloning and characterization of αTC6 cells has been described elsewhere.[19] The cells secrete large amounts of glucagon and no insulin in 16.5 mmol/L glucose.[19] αTC6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/L glucose supplemented with 10% fetal calf serum, penicillin (100 IU/mL), and streptomycin (100 μg/mL) under humidified atmosphere of 5% CO_2 95% air at 37°C. PC12 cells[20] and human aortic endothelial cells[21] were cultured as previously described.

Cell Perifusion Experiment

αTC6 cells were mechanically harvested and submitted to perifusion experiment.[22] Briefly, 2.5×10^6 viable cells, as determined by trypan blue exclusion, were placed in a small Sephadex-G25 chamber, kept at 37°C, and perifused at a constant flow rate of 0.33 mL/min by using a peristaltic pump. The perifusion medium was Krebs-Ringer bicarbonate buffer, pH 7.4, containing 0.1% bovine serum albumin (BSA) and 10 mmol/L glucose (KRBG), which was equilibrated with 95% O_2 5% CO_2, and immersed in a water bath at 37°C throughout the experiment. After a 60-minute equilibration, the effluent perifusate was collected in 5-minute fractions (1.65 mL/fraction) into tubes containing 1,000 U aprotinin. Test substances dissolved with KRBG were infused into the chamber by using an infusion pump. The effluent was stored at −20°C until assayed for glucagon and nitrite.

Static Experiment

For glucagon release experiments, the cells were plated in 24-well dishes at a density of 10^5 cells/well. Three to four days after plating, the culture medium was aspirated, and the cells were preincubated in serum-free DMEM for 24 hours followed by a 15-minute incubation in fresh DMEM in the presence or absence of test substances. The

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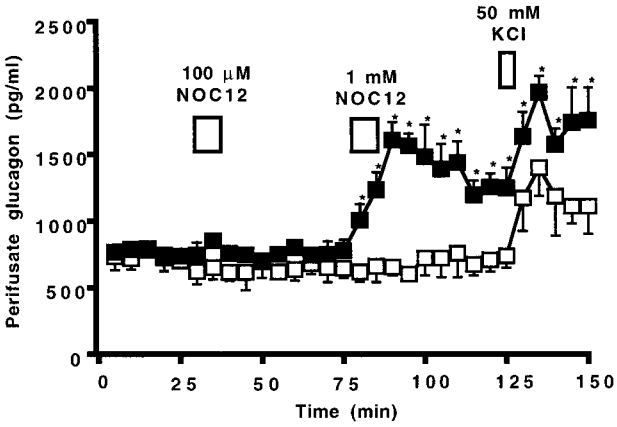

Fig 1. Effect of NOC12 on glucagon secretion from perifused αTc6 cells. NOC12 (100 μmol/L and 1 mmol/L) (■) or KRBG (□) as a control was applied as a 10-minute pulse. The cells were depolarized with 50 mmol/L KCl (5 minutes) to verify the cell viability. Glucagon concentration in each 5-minute fraction was assayed by RIA. Data represent mean ± SE values of 4 separate experiments. SE bars smaller than the size of symbol were omitted. * *P* < .05 *v* control.

incubations were performed at 37°C under 5% CO_2 95% air. The reactions were terminated by aspirating the incubation medium. The collected samples were mixed with 500 U of aprotinin and stored at −20°C until assayed for glucagon.

For determination of intracellular cGMP accumulation, αTc6 cells cultured in 100-mm dishes were preincubated in Krebs-HEPES solution, pH 7.4, at 37°C for 20 minutes in the presence or absence of IBMX (1 mmol/L). NOC12 at a final concentration of 1 mmol/L was added, and the cells were further incubated for 15 minutes at 37°C under 5% CO_2 95% air. cGMP was then extracted in 0.1 N HCl on ice for 30 minutes. The extract was neutralized with one tenth volume of 1 N NaOH and stored at −70°C until assayed for cGMP.

Determination of NOS Activity

NOS activity was measured by production of L-[^{3}H] citrulline from L-[^{3}H] arginine as previously described.[20] Supernatant (50 μL) of cell lysate was incubated at 37°C for 20 minutes in the presence of 50 mmol/L Tris · HCl (pH 7.5), 1 mmol/L nicotinamide adenine dinucleotide phosphate (NADPH), 10 μmol/L flavin adenine dinucleotide, 10 μmol/L tetrahydrobiopterin, 40 μmol/L L-arginine, 3.7 MBq L-[^{3}H] arginine, and 4 mmol/L $CaCl_2$ in a total volume of 100 μL. The reaction was halted by the addition of 1.25 mL ice-cold 20 mmol/L HEPES (pH 5.5), and the total volume was applied on a Dowex-50W × 8 (200 to 400 mesh, Na^+ form) column (Sigma, St Louis, MO) preequilibrated with 20 mmol/L HEPES (pH 5.5). The flow-through and 2-mL wash of distilled water were collected, and the radioactivity was measured by a liquid scintillation counter. The recovery rate of citrulline determined by applying L-[^{14}C] citrulline solution was 80% to 90%. The L-[^{3}H] citrulline concentration produced was corrected by the recovery rate.

Assay

Perifusate and medium concentrations of glucagon (Daiichi Radioisotope Institute, Tokyo, Japan) and cGMP levels (Amersham International Plc, Buckinghamshire, UK) in cell extracts were determined by commercial radioimmunoassay (RIA) kits according to the manufacturers' recommendations. Insulin concentrations were measured by RIA as previously described.[23] Perifusate nitrite concentration was assayed as previously described.[24] The minimal detectable concentration was 1.56 μmol/L when the standard was prepared with the perifusion medium. Protein content was measured using Bio-Rad (Richmond, CA) protein assay kit with BSA as the standard.

Statistics

All results are expressed as mean ± SE. The statistical difference was evaluated by analysis of variance and Fisher's test. A *P* value less than .05 was considered significant.

RESULTS

As shown in Fig 1, NOC12 (1 mmol/L), an NO donor, elicited an increase in glucagon release from perifused αTc6 cells. After a prompt response, the perifusate glucagon level gradually decreased, but was still elevated at 125 minutes. A lower concentration of NOC12 (100 μmol/L) did not significantly increase the glucagon release. Depolarization with 50 mmol/L KCl resulted in comparable increments of perifusate glucagon concentration in NOC12-treated and control cells. As shown in Fig 2, treatment with NOC12 (1 mmol/L) resulted in a 2.5-fold increase in medium glucagon concentrations in static incubation. Rp-cGMPS (100 μmol/L), an inhibitor of cGMP-dependent protein kinase, inhibited NOC12-induced glucagon release. Rp-cGMPS did not affect the basal glucagon secretion. The effect of NOC12 on cGMP accumulation in αTc6 cells is shown in Fig 3. NOC12 (1 mmol/L) elicited an increase in cellular level of cGMP. When the cells were pretreated with IBMX, the response was greater than that seen in untreated cells. Figure 4 illustrates the effect of 8-Br-cGMP on glucagon secretion. The permeable cGMP agonist increased glucagon release in a dose-dependent manner. The highest concentration

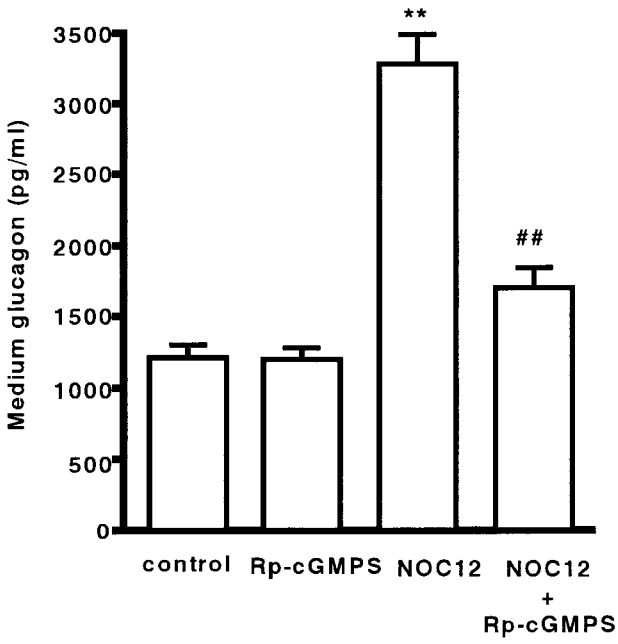

Fig 2. Effect of NOC12 and Rp-cGMPS on glucagon secretion from αTc6 cells in static incubation. αTc6 cells were incubated in DMEM at 37°C for 15 minutes in the presence or absence of NOC12 (1 mmol/L) and Rp-cGMPS (100 μmol/L). Data are shown as mean ± SE of 6 samples. ** *P* < .01 *v* control. ## *P* < .01 *v* NOC12 alone.

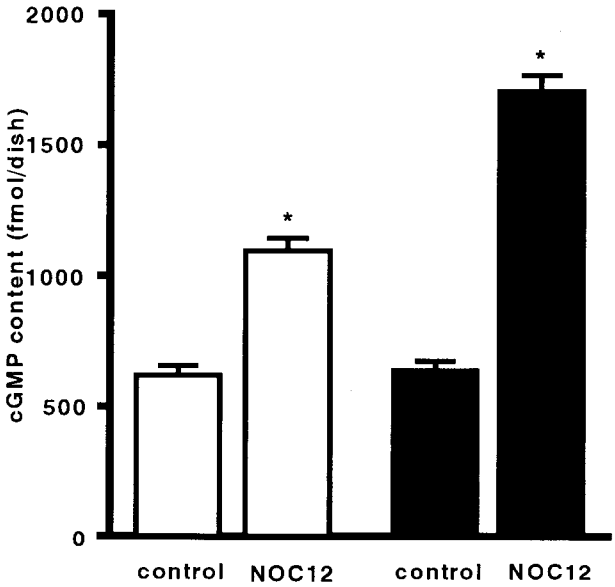

Fig 3. Effect of NOC12 on cGMP accumulation in αTc6 cells. The cells were cultured in 100-mm dishes and preincubated in Krebs-HEPES solution at 37°C for 20 minutes in the presence (closed bar) or absence (open bar) of IBMX (1 mmol/L). NOC12 at a final concentration of 1 mmol/L was added followed by a 15-minute incubation. IBMX was present also during the incubation period. Control dishes received Krebs-HEPES solution as a vehicle. Cellular cGMP content was assayed by RIA. Shown are mean ± SE values of 4 dishes. * *P* < .001 *v* control.

(1 mmol/L) evoked approximately a 2-fold increase in medium glucagon concentrations. Insulin release at the basal and stimulated states was under the minimal detectable level (0.2 ng/mL) both in perifusion and static incubation experiments.

L-arginine (15 mmol/L) increased glucagon release from perifused αTc6 cells, which was inhibited by L-NAME in a dose-related manner (Figs 5 and 6). Again, insulin levels were undetectable. Addition of KRBG did not affect glucagon release (data not shown). The basal nitrite level was under the minimal detectable concentration and remained undetectable throughout the depolarization-induced glucagon release. Addition of L-arginine also resulted in an increase in the medium nitrite level with a peak (3.68 ± 0.56 μmol/L, n = 4) in the same fraction as glucagon (45 minutes). The nitrite level was under the minimal detectable concentration in the presence of L-NAME.

NOS activity in αTc6 cells determined by L-citrulline formation assay was 325 ± 12 nmol/mg protein/20 min (n = 4). In comparison, those in PC12 and human aortic endothelial cells were 0.40 ± 0.02 nmol/mg protein/20 min and 6.13 ± 1.00 μmol/mg protein/20 min, respectively.

DISCUSSION

There has been direct and indirect evidence suggesting that NO might play a stimulating role in glucagon secretion. It was reported that hydroxylamine, an intracellular NO donor, stimulated glucagon release in isolated mouse islets in vitro.[12,15] L-NAME, an NOS inhibitor, inhibited depolarization- and carbachol-induced glucagon secretion in the presence of L-arginine in the islets in vivo and in vitro.[12,15] Recently, Hennings-son et al[25] showed that exogenous NO gas inhibited insulin and stimulated glucagon release from isolated mouse islets. When interpreting these observations in experiments using isolated islets, however, one must be careful to note that the β cells are also contained in the tissue. Insulin is reportedly an inhibitor of glucagon release,[17,18] and NO is a negative regulator for insulin release in the islets.[7,9-12,25] Thus, a decrease by NO in the inhibitory effect of insulin could itself cause an increase in glucagon secretion. To exclude this possibility, we used clonal αTC6 cells.[19] These cells lack proinsulin mRNA, although the original αTC1 line produces a small quantity of insulin.[19] In the present study, we clearly showed that 1 mmol/L NOC12, an NO donor,[26] increased glucagon release from αTC6 cells both in static incubation and cell perifusion experiments in the absence of detectable insulin. This is the first line of evidence showing that NO donors stimulate glucagon release in the absence of insulin-secreting cells.

Stimulation of a soluble form of guanylate cyclase and subsequent formation of cGMP are involved in NO action.[2,3,27] Increased levels of cGMP may activate cGMP-dependent protein kinase and activate ion channels.[1] NOC12-induced glucagon release from αTc6 cells was suppressed by Rp-cGMPS, an inhibitor of cGMP-dependent protein kinase. This finding suggests that the effect of NO on glucagon release is mediated, at least partly, through a cGMP-dependent pathway in these cells. In fact, we showed that glucagon release from αTc6 cells was dose-dependently increased by 8-Br-cGMP, a permeable analog of cGMP in the present study. The response to the maximal concentration (1 mmol/L) of 8-Br-cGMP was similar to the

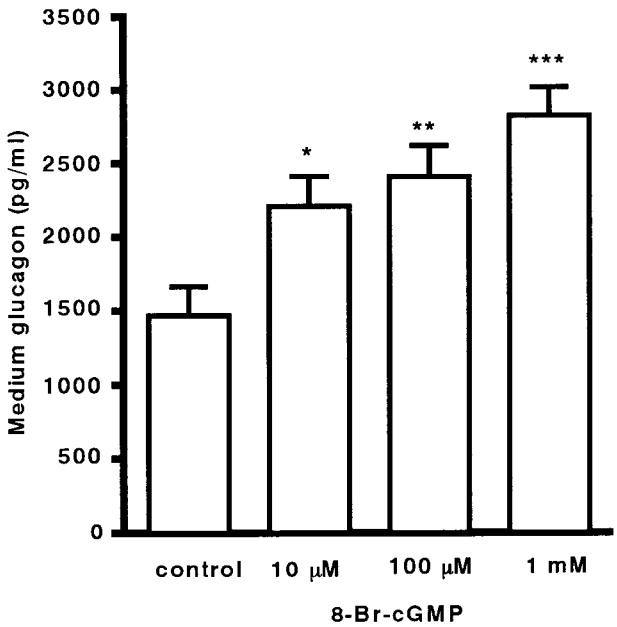

Fig 4. Effect of 8-Br-cGMP on glucagon secretion from αTc6 cells in static incubation. αTc6 cells were incubated in DMEM at 37°C for 15 minutes in the presence of an increasing dose of 8-Br-cGMP (10 μmol/L, 100 μmol/L and 1 mmol/L). Data are shown as mean ± SE of 6 samples. * *P* < .05, ** *P* < .01, * *P* < .001 *v* control.**

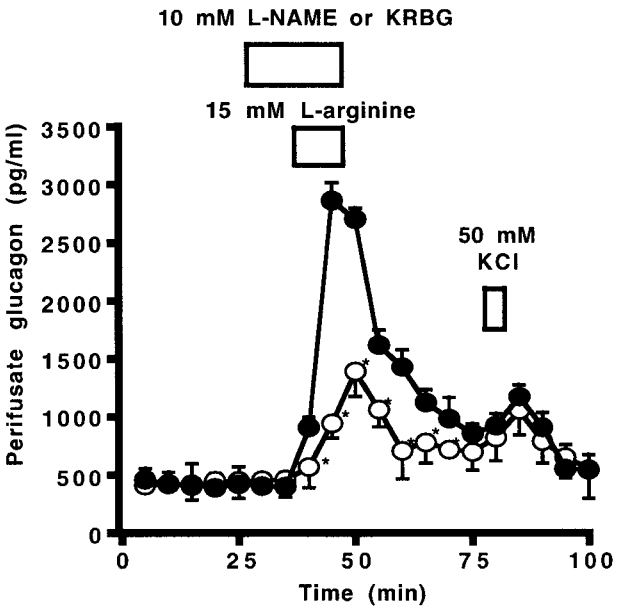

Fig 5. Effect of L-NAME on glucagon release induced by L-arginine from perifused αTc6 cells. L-arginine (15 mmol/L) was applied as a 10-minute pulse in the presence of control perifusion medium (●) or medium containing L-NAME (10 mmol/L) (○). Glucagon concentration in each 5-minute fraction was shown. Data represent mean ± SE of 4 separate experiments. SE bars smaller than the size of symbol were omitted. * *P* < .05 *v* control.

response to NOC12. In addition, NOC12 at a concentration of 1 mmol/L elicited an increase in the cellular content of cGMP, as well as glucagon release. Panagiotidis et al[9] failed to show that 3 μmol/L hydroxylamine elicited an increase in cellular content of cGMP in isolated mouse islets, although hydroxylamine at the same concentration increased glucagon secretion.[12] It is possible that an increase in cGMP levels in the glucagon cells was not detected in whole islets because the cells comprise only 15% to 20% of the cell population.[12]

In the present study, we observed that L-arginine elicited an increase in glucagon release from αTc6 cells. One possible mechanism accounting for this response is stimulation by NO derived from L-arginine through the action of NOS, which was shown by the L-citrulline formation assay in these cells. To support this possibility, L-NAME inhibited L-arginine–induced glucagon release in a dose-related manner. In addition, L-arginine increased perifusate nitrite concentration to 3.68 ± 0.56 μmol/L. We previously observed that nitrite concentration in static incubation medium of GH_3 somatomammotroph cells was 0.1 to 0.2 μmol/L.[24] This level of NO was biologically effective in GH_3 cells because L-NAME and oxyhemoglobin stimulated GH secretion by inhibiting NO synthesis and by trapping NO, respectively.[28]

Several lines of evidence suggest that the generation of NO from L-arginine in αTc6 cells reflects a physiologic event in normal α cells in the pancreatic islet. Biochemical studies on mouse islet NOS showed dependence on Ca^{2+} and calmodulin,[15,16] properties of the constitutive NOS isoforms (cNOS). Immunohistochemical studies showed the presence of cNOS in the pancreatic islet of the rat[4,29] and the mouse.[6,7] Although it has been consistently shown that cNOS activity is located in β cells, there have been conflicting reports as to its occurrence in normal glucagon-releasing cells.[5,25] Recently, Alm et al[30] showed immunoreactivity for cNOS in glucagon- as well as insulin-immunoreactive rat islet cells using immunocytochemical confocal microscopy. Thus, it is possible that L-arginine–derived NO might play a stimulating role in glucagon secretion as a paracrine or autocrine transmitter molecule in the islets of Langerhans.

In this study, glucagon release from perifused αTc6 cells showed a sustained response to NOC12 with a peak value of 2.5-fold of control. The response to L-arginine was quite different from that to NOC12, namely, a sharp increase with a peak value of 5-to 6-fold of control. Mechanisms accounting for distinct responses of αTc6 cells to NOC12 and L-arginine remain to be elucidated. A metabolic fuel effect and depolarization of the plasma membrane by uptake of the positively charged L-arginine molecules have also been proposed as possible mechanisms of the stimulatory effect of L-arginine on glucagon secretion. These possibilities were not thoroughly examined in this study. Henningsson et al[15] found that L-homoarginine, an L-arginine analog not metabolized by arginase within islet tissues,[31] was equipotent in stimulating glucagon release from isolated mouse islets, suggesting that the effect of L-arginine was independent of any metabolic fuel effect. L-arginine–induced glucagon release was inhibited by NOS inhibitors in depolarized mouse islets.[10] Åkesson et al[11] showed D-arginine did not mimic the inhibitory effect of

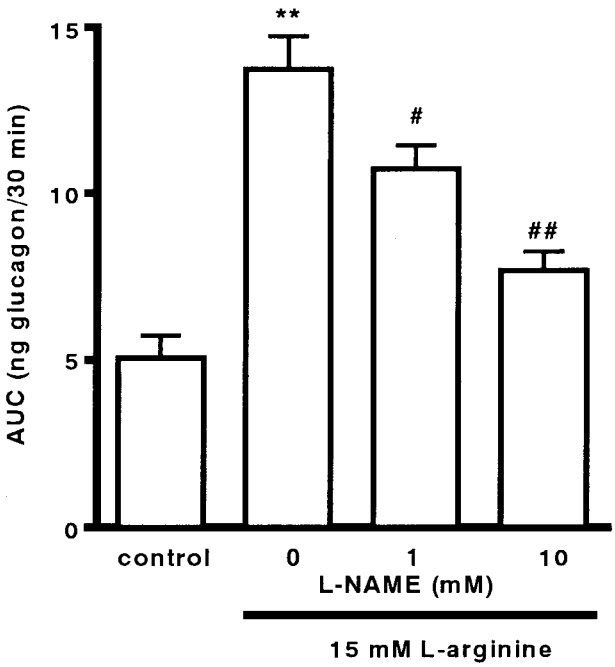

Fig 6. Inhibition by L-NAME (1 and 10 mmol/L) of L-arginine (15 mmol/L)–induced glucagon secretion from perifused αTc6 cells. Mean ± SE values (n = 4) of AUC values during 30 minutes (fractions 8 to 13 in Fig 5) are shown. ** *P* < .01 *v* control; # *P* < .05, ## *P* < .01 *v* L-arginine alone.

L-NAME on L-arginine–induced glucagon secretion in mice in vivo. These findings argue against the possibility that a charge-induced depolarization could play a major role in L-arginine–induced glucagon secretion. In mouse islet β cells, L-NAME stimulated insulin release by directly blocking ATP-regulated K^+ (K_{ATP}) channels,[32] which were recently shown to be expressed in rat pancreatic α cells, as well.[33] The effect of L-NAME on K_{ATP} channels on αTc6 cells, if any, would be masked by the NOS-inhibiting activity, because we observed that L-NAME inhibited L-arginine–induced glucagon secretion in these cells.

In conclusion, our present findings suggest that NO plays a stimulating role on glucagon release in α cells, and that a cGMP-dependent pathway is involved in NO action. These findings also provide further evidence that L-arginine might play a stimulating role in the regulation of glucagon secretion, at least partly, through generation of NO in the islets.

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