Korean J. Zool. 36:433-438, 1993

Changes in the Cellular cGMP Levels and Guanylate Cyclase Activities during Chick Myoblast Fusion

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In the previous paper (Choi *et al.*, 1992), we found that a large but transient elevation in intracellular cGMP levels occur concomitant with the myoblast fusion. To establish the physiological significance of the elevation of cGMP levels, the change in guanylate cyclase activity during myoblast fusion and the correlation between various chemicals that may affect guanylate cyclase activity and myoblast fusion were examined.

Sodium nitroprusside, a nitric oxide-forming compound, induced a precocious fusion and increased guanylate cyclase activity compared to the control. Furthermore, L-N^G-monomethyl arginine, specific inhibitor of L-arginine: nitric oxide synthase, inhibited the cell fusion in a dose-dependent manner, without affecting biochemical differentiation.

On the basis of our present findings, we propose that the onset of myoblast fusion is somehow correlated with the rise in cellular cGMP levels that is regulated by the activation or inhibition of soluble guanylate cyclase, via as yet undefined mechanism but possibly through L-arginine: nitric oxide pathway.

KEY WORDS: cGMP, Guanylate cyclase, Myoblast fusion

During the differentiation of skeletal myoblasts, the cells divide, align in arrays, and undergo membrane fusion leading to cytoplasmic continuity (Knudsen and Horwitz, 1977). On the time of fusion process, prostaglandin E_1 (PGE_1) induces an increased Ca^{2+} influx which triggers the fusion (David *et al.*, 1981a, 1981b). This Ca^{2+} influx is found to be tightly linked to a transient elevation in cGMP levels in chick myoblast differentiation (Choi *et al.*, 1992). It has been demonstrated that several agents can enhance cGMP accumulation in a neuronal cell line as a result of activation of guanylate cyclase (Reiser *et al.*, 1990). These findings suggest that the rise of cGMP levels may be linked to the change of guanylate cyclase activity in myoblast fusion.

Guanylate cyclase has been found in a number of tissues throughout the animal kingdom and the enzyme activity has been generally found in soluble and particulate fractions of tissues (Kimura and Murad, 1976). Guanylate cyclase has shown different responsiveness to its activators such as nitric oxide (NO)-forming compounds in various tissue preparations (Arnold *et al.*, 1977). The studies utilizing guanylate cyclase activator, sodium nitroprusside (SNP), have demonstrated that NO formed is proportional to the rate of Ca^{2+} influx and that NO elevates intracellular cGMP levels (Pandol and Schoeffield-Payne, 1990). For instance, NO is induced by bradykinin and calcium ionophore A23187 in the endothelial cell (Palmer *et al.*, 1988). However, this NO stimulation of guanylate cyclase is blocked by methylene blue (MB), guanylate cyclase inhibitor (Wolin *et al.*, 1990).

Furthermore, NO is synthesized from the

terminal guanidino nitrogen atom of L-arginine through L-arginine:nitric oxide synthase (NOS), which is Ca^{2+}-dependent and is inhibited by L-N^G-monomethyl arginine (L-NMMA) (Palacious *et al.*, 1988). This biochemical system, called L-arginine: NO pathway, appears to represent a widespread signal transduction mechanism with guanylate cyclase as an effector system (Moncada *et al.*, 1989). These reports suggest that NO may be involved in a mechanism by which NO triggers the rise of intracellular cGMP levels, which in turn may lead to myoblast fusion.

In the present study, we demonstrate that the correlation between the activity of guanylate cyclase and the rise of cGMP levels in myoblast fusion and that the possible involvement of NO in triggering the rise in guanylate cyclase activity.

Materials and Methods

Materials

Eagle's minimum essential medium (MEM), horse serum, and antibiotics were obtained from Gibco Laboratories. Tissue culture dishes were from Nunc, and Na[^{125}I] was from Amersham. MB, SNP, cGMP antibody, L-NMMA and other reagents were from Sigma Chemical Co.

Myoblast cultures

Chick myoblasts were obtained from the breast muscles of 12-day-old chick embryos (Tepperman *et al.*, 1975). The cells were plated at a density of 1×10^5 cells/ml in MEM supplemented with 10 % horse serum and 2 % embryo extracts at pH 7.2-7.4 and cultured in a humidified atmosphere of 5 % CO_2 and 95 % air at 37°C.

Measurement of cell fusion

Cells were fixed with 1 % glutaraldehyde and stained with Giemsa for the estimation of percent fusion. Cell fusion was estimated by direct microscopic examination at a magnification of 250 X. Cells were considered fused only if there were clear cytoplasmic continuity and at least three nuclei were present in each myotube.

Drug additions

SNP was prepared as 50 mM stock solution in distilled water, and was serially diluted to appropriate concentration prior to use. L-NMMA was prepared as 20 mM stock solution in distilled water.

Enzyme solution preparation

Cells were harvested and homogenized in ice-cold 50 mM Tris-HCl buffer, pH 7.5. After centrifugation at 20,000 $\times g$ for 30 min, the homogenate was separated into soluble and particulate fractions by centrifugation at 100,000 $\times g$ for 60 min. All subsequent procedures for preparing the enzyme were carried out at 4°C. Particulate fraction was suspended in a volume of buffer equal to that of original homogenate. Solubilization of 100,000 $\times g$ precipitate was accomplished by incubation with 1 % Nonidet P-40 for 60 min at 4°C. Preparations were centrifuged at 100,000 $\times g$ for 60 min prior to guanylate cyclase assay (Kimura and Murad, 1974).

Guanylate cyclase activity determination

Assay mixture for guanylate cyclase activity contained 50 mM Tris-HCl buffer, pH 7.6, 1 mM GTP, 1 mM 1-methyl-3-isobutylxanthine, 3 mM divalent cation ($CaCl_2$, $MgCl_2$, or $MnCl_2$), 20 μg of creatine kinase (120 to 135 units per mg), 15 mM creatine phosphate, and enzyme (10 to 100 μg of protein) in a final volume of 100 μl. The reaction was started by adding the enzyme and incubated at 37°C for 10-20 min. To prevent nonenzymatic formation of cGMP, reaction was terminated by adding 0.9 ml of 50 mM sodium acetate buffer (pH 4.0), and heated for 3 min at 90°C . An aliquot (50 to 150 μl) of diluted incubation extract was used directly for the radioimmunoassay of cGMP (Kimura and Murad, 1974).

Cyclic GMP assay

Cells seeded on 60-mm dishes were harvested by adding 0.5 ml of 50 mM sodium acetate buffer (pH 5.2) which was preheated to 90°C. After incubation at 90°C for 1 hr, the tube was subjected to vigorous vortexing for 2 min, followed

by incubation at 0-5°C for 30 min. Samples were stored at -70°C and assayed within three days. Immediately prior to assay, the samples were thawed and cell debris was removed by centrifugation at 12,800 $\times g$ at 4°C for 10 min. An aliquot (100 μl) was acetylated and the cGMP content was determined by the radioimmunoassay of Steiner *et al.* (1972). The cGMP content was normalized to the number of nuclei.

Acetylcholine receptor assay

Acetylcholine receptors were assayed as described by Lee and Tseng (1966). 10 nCi/ml [^{125}I] α-bungarotoxin was added to multi-well plates 56 hr after plating and incubated for 1 hr. The cells were rapidly rinsed four times at 3 min intervals with MEM. The cells were harvested with 4 % SDS and counted for radioactivity in a γ-counter.

Assay of DNA synthesis

0.1 μCi [^{3}H]thymidine was added to multi-well plate at 24 hr after plating and incubated for 33 hr. The cells were rinsed three times with phosphate buffered saline at room temperature and then rinsed twice with 5 % TCA. After 10 min, the cells were dissolved in 100 % TCA and the radioactivity in them was counted in a liquid scintillation counter.

Results

Stimulation of myoblast fusion and rise in cGMP levels by SNP

To explore the mechanism by which the cGMP levels rise at time of cell fusion, SNP was treated to myoblast cultures 24 hr after plating. SNP stimulated myoblast fusion in a dose-dependent manner up to 30 μM and the myoblasts exposed to 30 μM SNP 47 hr after plating resulted in a precocious fusion (Fig. 1). The elevation of cGMP levels elicited by SNP was again dose-dependent up to 30 μM (Fig. 2). Thus, it appears that myoblast fusion is tightly linked with the cGMP levels.

Rise of guanylate cyclase activity by SNP

It is quite probable that the elevation or depression of cGMP levels is caused by the activation or inhibition of guanylate cyclase. Therefore, we then examined if SNP really affect guanylate cyclase activity in the cell extracts. Since guanylate cyclase activity is highly related with divalent cations, guanylate cyclase assay was performed in the presence of 3 mM Mg^{2+}, with an intention to have adequate activities to be easily measurable. As expected, guanylate cyclase

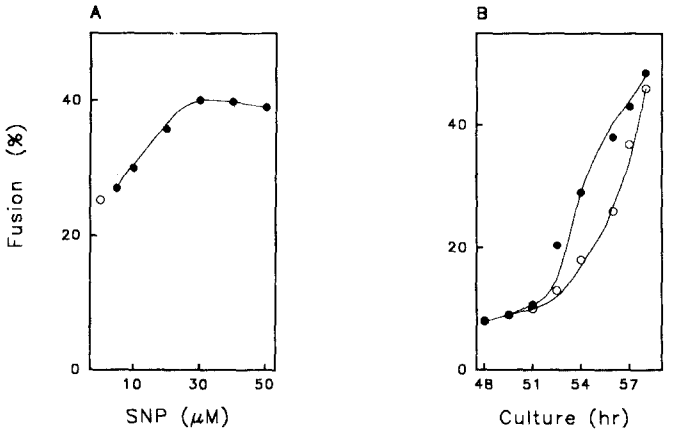

Fig. 1. (A) Dose-dependent stimulation of myoblast fusion by SNP up to 30 μM. Cells were exposed to various doses of SNP at 47 hr and scored for percent fusion 6-7 hr after the treatment. Control level is shown for comparison (○). (B) 30 μM SNP produced a precocious fusion in myoblasts in culture. ○-○, control; ●-●, treatment. Each point represents the mean of triplicate determinations.

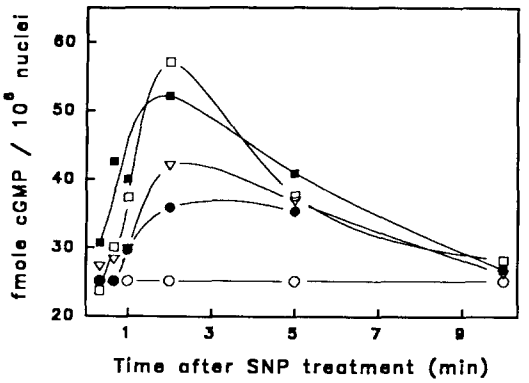

Fig. 2. Dose-dependent rise in the cGMP levels up to 30 μM by SNP. At 50 hr after plating, the cells were washed twice with 2 ml MEM. Following 1-hr equilibration, medium was aspirated and the cultures were preincubated in 3 ml MEM, containing 25 mM Hepes, 2 mg/ml bovine serum albumin for 15 min at 37°C. SNP was then added to the culture medium and incubated at 37°C. After incubation for indicated times, the cells were harvested for RIA. ○, control; ●, 10 μM; ▽, 20 μM; □, 30 μM; ■, 50 μM SNP.

activity was elevated by SNP compared to the control (Fig. 3). Thus, it appears that SNP affects myoblast fusion through mediating guanylate cyclase activity.

Inhibition of myoblast fusion by L-NMMA

To assess more specifically the nature of guanylate cyclase effect on myoblast fusion, L-NMMA was employed. L-NMMA was found to

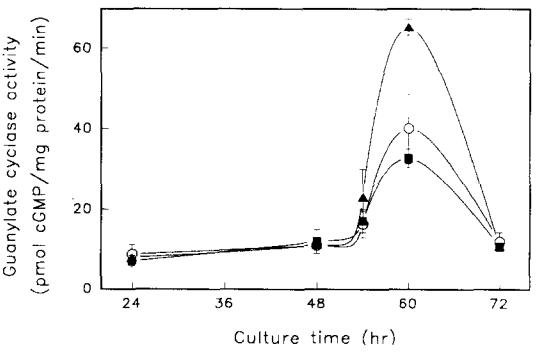

Fig. 3. Effects of SNP and MB on the guanylate cyclase activities during myoblast cultures. Guanylate cyclase activities were assayed in cell homogenates in the presence of 3 mM Mg^{2+}. ○, control; ▲, SNP; ■, MB.

have a profound effect on myoblast fusion. The cells treated with L-NMMA (100 μM) 24 hr after plating delayed and decreased the cell fusion (Fig. 4). It seems likely that L-NMMA decreases myoblast fusion by inactivating guanylate cyclase through decreased formation of NO. Then, we studied the effects of L-NMMA on myoblast fusion and biochemical differentiation. No difference was observed in the rate of [^{3}H]thymidine incorporation of either control or L-NMMA-treated cultures. Similarly, [^{125}I]α-bungarotoxin binding to either control or L-NMMA-treated cultures was also the same (Table 1).

Discussion

Our previous report that a transient rise in the cGMP levels, which is closely associated with the Ca^{2+} influx, occurs concomitant with the onset of myoblast fusion (Choi *et al.*, 1992) led us to examine further the significance of the cGMP peak in myoblast fusion. The first attempt, therefore, was to adopt guanylate cyclase activator to observe their possible effects on myoblast fusion and on cellular cGMP levels.

As exprected, myoblast fusion was stimulated by SNP in a dose-dependent manner up to 30 μM

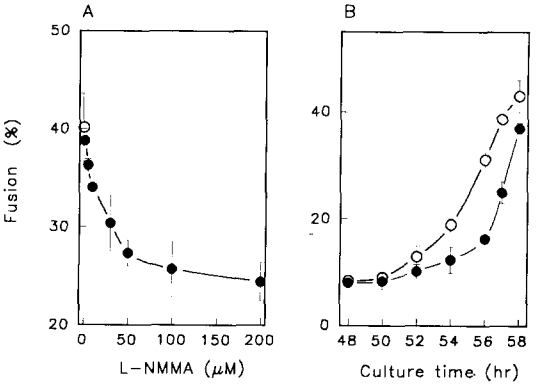

Fig. 4. (A) Dose-dependent inhibition of myoblast fusion by L-NMMA. Cells were exposed to various doses of L-NMMA 24 hr after plating and incubated for 30 hr. Control level is shown for comparison (○). (B) Exposure of cells to 100 μM L-NMMA 24 hr after plating produced a significant retardation in the fusion. ○-○, control; ●-●, L-NMMA.

Table 1. Effect of L-N^G-monomethyl arginine on the myoblast fusion and biochemical differentiation.

Assays	Control	L-NMMA
Fusion (%)	38.7 ± 1.1	25.1 ± 2.9
[^{3}H]Thymidine incorporation (cpm/well)	11941 ± 1024	12026 ± 890
[^{125}I]α-Bungarotoxin binding (cpm/well)	1371 ± 243	1376 ± 68

Cells were exposed to 100 μM L-NMMA 24 hr after plating and harvested at 33 hr after the treatment. Each value represents the mean ± SD.

(Fig. 1A) and 30 μM SNP treated at 47 hr after plating resulted in a precocious fusion in myoblast cultures (Fig. 1B). Furthermore, SNP treated at various concentrations at 51 hr after plating caused the elevation of cGMP levels within 2 min, again 30 μM SNP being the most effective (Fig. 2). This is in contrast with the previous fnding that myoblast fusion was inhibited by MB in a dose-dependent manner up to 0.6 μM. Furthermore, cellular cGMP levels are also depressed by MB (0. 6 μM) in time of appearance and size of the peak as well (Choi *et al.*, 1992). Taken together, it is quite probable that the correlation between myoblast fusion and cellular cGMP levels exists.

To examine if myoblast fusion is really related with the change in cGMP levels and whether guanylate cyclase activator and inhibitor really regulate cellular cGMP levels, we have investigated the effect of SNP and MB on guanylate cyclase activity in cell homogenates. Guanylate cyclase in cell homogenates was indeed activated by SNP, demonstrating the presence of soluble guanylate cyclase in myoblasts (Fig. 3). In contrast, the guanylate cyclase was depressed by MB that is generally assumed to be an inhibitor of activation of the soluble form of guanylate cyclase. However, the mechanism of inhibition of guanylate cyclase activation by MB remains to be established. On the contrary, the mechanism whereby SNP or those compounds, capable of forming NO, activate guanylate cyclase is likely to work through a similar but undefined mechanism (Arnold *et al.*, 1977).

Currently, it is accepted that the L-arginine:NO pathway is the endogenous activator of soluble guanylate cyclase and is a widespread mechanism for regulating cell function and communication (Moncada *et al.*, 1989). Thus, next investigation has been focused on the effect of L-NMMA, specific NOS inhibitor, on myoblast fusion and biochemical differentiation. Dose-dependent inhibition of myoblast fusion was observed by L-NMMA treated 24 hr after plating and incubated for 34 hr. In addition, the retardation of time course of myoblast fusion resulted by L-NMMA treatment 24 hr after plating (Fig. 4B). In contrast, L-NMMA was without effect on biochemical differentiation (Table 1). For example, [^{125}I]α-bungarotoxin binding and acetylcholine receptor immunoblotting revealed no difference between control and L-NMMA treated cultures. Thus, the effect of L-NMMA is limited only on the myoblast fusion but not on the biochemical expression. The effect of L-NMMA is quite similar to that of MB in that it only affects myoblast fusion but not biochemical differentiation (Choi *et al.* 1992).

In conclusion, we propose that the onset of myoblast fusion is somehow correlated with the rise in cellular cGMP levels that is regulated by the activation of soluble guanylate cyclase, possibly via L-arginine:NO pathway.

Acknowledgments

This work was supported by grant from Korea Science and Engineering Foundation through SRC for Cell Differentiation.

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(Accepted July 2, 1993)

근원세포 융합시 Cellular cGMP 수준과 Guanylate cyclase 활성의 변화

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본 연구를 통해서 근세포 융합과정에서 신호 전달물질의 가능성이 제기되고 있는 세포내 cGMP peak가 guanylate cyclase activity의 변화와 관련이 있으며, guanylate cyclase는 L-arginine: NO synthase에 의해서 촉진될 것임을 입증할 수 있는 간접적 증거를 제시한다. 즉, SNP는 근세포의 융합과 guanylate cyclase activity를 아울러 증가시키며, L-arginine: NO synthase inhibitor인 L-N^G-monomethyl arginine 은 biochemical differentiation에는 영향을 주지 않고 근세포 융합만을 억제한다. 이러한 결과들과 methylene blue가 근세포의 융합을 억제하면서도 biochemical differentiation에는 영향을 주지 않으며 guanylate cyclase activity를 억제하는 사실들을 종합해서 생각할 때, 근세포 융합에서 cGMP peak가 guanylate cyclase activity의 활성화와 관련이 있으며, L-arginine: NO synthase가 Ca^{2+} influx와 guanylate cyclase 사이를 매개할 가능성을 암시한다.