Nitric Oxide as a Messenger Molecule for Myoblast Fusion*

(Received for publication, January 31, 1994)

Kun Ho Lee, Mi Yeong Baek, Kyung Yeop Moon, Woo Keun Song, Chin Ha Chung, Doo Bong Ha, and Man-Sik Kang

Department of Molecular Biology and SRC for Cell Differentiation, College of Natural Sciences, Seoul National University, Seoul 151-742, Korea

Nitric oxide (NO) is a messenger molecule of vascular endothelial cells, macrophages, and neurons. Here, we demonstrate that the activity of NO synthase increases transiently but dramatically in chick embryonic myoblasts that are competent for fusion. This activity requires Ca++, calmodulin, and NADPH. In addition, the increase in NO synthase activity coincides with an increase in cellular cGMP level. Furthermore, NO generated by treatment with sodium nitroprusside induces precocious myoblast fusion, while treatment with N(-monomethyl-l-arginine, a competitive inhibitor of NO synthase, or methylene blue, an inhibitor of guanylate cyclase, delays fusion. These results provide the first evidence for a strong association of NO with myoblast fusion.

NO is a messenger molecule that mediates a variety of cellular functions (Snyder, 1982; Moncada et al., 1991;Ignarro, 1990). In blood vessels, NO relaxes smooth muscle by stimulating formation of cGMP through activation of soluble guanylate cyclase (Ignarro and Kadowitz,1985; Moncada et al.,1988). In the brain, NO mediates the actions of the excitatory neurotransmitter glutamate also by elevating cGMP concentration (Bredt and Snyder, 1989; Knowles et al., 1989; Izumi et al., 1992). It is also responsible for the bactericidal and tumoricidal effects of macrophages and leucocytes (Hibbs et al., 1988; Nathan and Hibbs, 1991). NO synthase (NOS) catalyzes the stoichiometric conversion of l-arginine to NO and l-citrulline (Bredt and Snyder, 1989; Kwon et al., 1990; Palmer and Moncada, 1989) and exists as at least two distinct isoforms (Bredt et al., 1991; Lamas et al., 1992; Lowenstein et al., 1992). NOS in endothelium and brain requires Ca++ and calmodulin (CaM) for activity and is constitutively expressed, while the enzyme in macrophages and leucocytes does not require the cofactors but is inducible (Kwon et al., 1990; Lowenstein et al., 1992; Bredt and Snyder, 1990; Busse and Mülisch, 1990).

*This work was supported by grants from Korea Science and Engineering Foundation through SRC for cell differentiation and grants from the Korean Ministry of Education. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed. Tel.: 82-2-880-6887; Fax: 82-2-872-1993.

The abbreviations used are: NOS, NO synthase; CaM, calmodulin; MEM, Eagle’s essential medium; NMMA, N(-monomethyl-l-arginine; SNP, sodium nitroprusside; MB, methylene blue; 8-BrcGMP, 8-bromo-cGMP.

A prominent event in the differentiation of embryonic muscle cells is the fusion of mononucleated myoblasts into multinucleated myotubes, and this myogenic process absolutely requires Ca++ influx (David et al., 1981; Wakalam, 1985; Entwistle et al., 1988; Przybylek et al., 1989). We have recently shown that cGMP concentration transiently but markedly increases just prior to myoblast fusion and that this increase is Ca++-dependent (Choi et al., 1992). This Ca++ requirement and subsequent increase in cellular cGMP level led us to examine the possibility that NO may act as a mediator for myoblast fusion.

EXPERIMENTAL PROCEDURES

Materials—Creatine kinase was purified from adult chick skeletal muscle as described (Eppenberger et al., 1967). Anti serum against creatine kinase was prepared by injecting the purified protein into albino rabbits. a-Bungarotoxin was radiiodinated using chloramine T (Hunter and Greenwood, 1962). Na[35S]51-[S]-protein A, and l-[guanido-14C]arginine (57.8 mCi/mmol) were purchased from Du Pont NEN. Culture media were obtained from Life Technologies, Inc, and all other reagents were purchased from Sigma.

Cell Cultures—Myoblasts were prepared from breast muscle of 12-day chick embryos as described (Kim et al., 1992). Briefly, the cells were plated on collagen-coated culture dishes at a concentration of 5 x 10^4 cells/ml in Eagle’s essential medium (MEM) containing 10% horse serum, 10% chick embryo extract, and 1% antibiotic/antimycotic solution. One day after the cell seeding, the culture medium was changed with the same medium but containing 2% embryo extract. Extent of myoblast fusion was expressed as the percentage of the number of nuclei in fused cells to the total number of nuclei in 10 randomly chosen fields under a phase contrast microscope. Cells containing more than three nuclei were regarded as the fused cells.

Assays—The activity of NO synthase was determined by monitoring the conversion of [14C]arginine to [14C]citrulline as described by Bredt and Snyder (1990). Reaction mixtures (0.2 ml) contained 50 mM HEPES (pH 7.4), 0.2 mM of l-[14C]arginine (57.8 mCi/mmol), 1 mM NADPH, 1 mM EDTA, 1.25 mM CaCl2, 10 mM pCaM, and appropriate amounts of the enzyme preparations. After incubation for 10 min at 37°C, the reactions were terminated by adding 1 ml of 10 mM EDTA. The samples were applied to 1 ml columns of AG50X-8 (Na+ form) and eluted with 2 ml of distilled, deionized water. Aliquots of the eluates ([14C]citrulline) were then counted for radioactivity.

Acetylcholine receptor was assayed by determining its ability to bind to a-Bungarotoxin (Lee and Tseng, 1966). Myoblasts that had been cultured for appropriate periods were treated with [3H]a-bungarotoxin (2 x 10^6 cpm/well) and incubated for the next 1 h. They were then rinsed with MEM, solubilized in 4% (w/v) sodium dodecyl sulfate (SDS), and counted for radioactivity.

RESULTS AND DISCUSSION

In order to determine whether primary cultures of chick embryonic myoblasts have any NOS activity, extracts were prepared from the cells and assayed for their ability to convert [14C]arginine to [14C]citrulline. A peak of NOS activity was found in the extracts obtained from myoblasts that are competent for fusion (i.e. cultured for 50–55 h) but not in rapidly proliferating myoblasts (for 24–36 h) and myotubes (for more than 72 h) (Fig. 1A). Thus, the appearance of NOS activity in cultured myoblasts seems to be differentiation time-dependent.

Extracts of breast muscle tissues were also prepared from variously aged chick embryos and assayed for their ability to convert [14C]arginine to [14C]citrulline. A peak of NOS activity was found in the extracts obtained from myoblasts that are competent for fusion (i.e. cultured for 50–55 h) but not in rapidly proliferating myoblasts (for 24–36 h) and myotubes (for more than 72 h) (Fig. 1B). In addition, little or no activity could be seen in the...
Homogenates were centrifuged the activation of NOS activity by CaM and NADPH could be the level of NOS activity in embryonic muscle tissues also appears been suggested (Brune et al., 1991; Bredt et al., 1992). Howev-er, it is also possible that the changes in the activity level is due to change during the course of myogenic differentiation. Regu-lation of the constitutive type of NOS by phosphorylation has been suggested (Brune et al., 1991; Bredt et al., 1992). How-ever, it is also possible that the changes in the activity level is due to alterations in the expression of NOS during the myogenic process.

The NOS activity in fusion-competent myoblasts required both Ca$^{2+}$ and CaM in addition to NADPH (Fig. 2). The basal activity seen alone or with NADPH or CaM appeared to be due to the presence of these cofactors in limited amounts in the extracts, because addition of EGTA or trifluoperazine, an antagonist of CaM, further reduced the basal activity. In addition, the activation of NOS activity by CaM and NADPH could be blocked by N$^{G}$-monomethyl-$
$-arginine (NMMA). Nearly identi-cal data were obtained with the muscle extracts from 13- to 14-day embryos. Therefore, the cofactor requirement of NOS activity in embryonic muscle cells resembles that of the constitutive, Ca$^{2+}$/CaM-dependent type of NOS.

The peak of NOS activity found in fusion-competent myo-blasts nearly coincided with the increase in intracellular cGMP level (Fig. 3A). Furthermore, treatment of the culture with NMMA inhibited the increase in cGMP level, suggesting that the marked and transient increase in NOS activity is responsible for the timely rise in cGMP level. Upon treatment of fusion-competent myoblasts with sodium nitroprusside (SNP), cGMP concentration rose and reached a maximal level at about 2 min and gradually decreased to a basal level by about 10 min (Fig. 3B). This effect of SNP was dose-dependent and was maximal at 30–50 μM. In addition, treatment with high concentrations of methylene blue (e.g. above 30 μM) blocked the increase in cGMP level even in the presence of SNP. Thus, NO appears to be responsible for cGMP formation through activation of guanylate cyclase in cultured myoblasts.

We then examined the effects of SNP and NMMA on myo-blast fusion. Increasing NO levels in the cell by SNP treatment induced precocious fusion, while inhibiting NO production by NMMA treatment delayed the time of fusion (Fig. 4A). Both effects were dose-dependent, and the effect by SNP was maximal at 30–50 μM (Fig. 4B), the same concentrations producing a maximal cGMP level (see above). At all concentrations tested, however, little or no effect was observed in myoblast proliferation as assessed by [H]thymidine incorporation. Microscopic analysis revealed that the cells treated with either agent aligned normally along their bipolar axes (data not shown). Furthermore, the synthesis of muscle-specific proteins including creatine kinase and the acetylcholine receptor was not affected by treatment with either SNP or NMMA (Table 1). Thus, the effect of NO is not due to simple toxicity but is specific for cell fusion. NMMA-mediated inhibition of myoblast fusion could be re-
versed by treatment with 8-Br-cGMP, an analog of cGMP, as well as SNP, but not by treatment with the Ca\textsuperscript{2+} ionophore A23187 (Table II). The inhibition of cell fusion by methylene blue could also be reversed by treatment with 8-Br-cGMP or high concentrations of SNP (e.g. 30 μM). Ca\textsuperscript{2+} influx is known to precede and be essential for myoblast fusion. Thus, it appears that increase in intracellular Ca\textsuperscript{2+} concentration causes an activation of myoblast NOS to produce NO, which in turn activates guanylate cyclase for cGMP formation. The role of cGMP in myoblast fusion remains unknown, but the addition of nanomolar concentrations of cGMP to the extract from fusion-competent myoblasts resulted in the specific phosphorylation of a 52-kDa protein.\textsuperscript{2} Further investigation of the phosphorylation of this protein or perhaps others may provide an insight into the mechanism for cGMP-mediated myoblast fusion.


Fig. 4. Effects of SNP and NMMA on myoblast fusion. A, cells cultured for 24 h were further cultured in the absence (A) and presence of 0.1 mM NMMA (○). The cells cultured for 47 h were also further cultured with 30 μM SNP (●). The extent of myoblast fusion was determined as in Fig. 1. B, cells cultured for 24 h and 47 h were treated with increasing amounts of NMMA (●) and SNP (●), respectively, and further cultured until 55–56 h. The extent of fusion was then determined. Similar data were obtained in three independent experiments.

TABLE I
Effects of SNP and NMMA on the expression of muscle-specific proteins in cultured myoblasts

<table>
<thead>
<tr>
<th>Treatment (concentration)</th>
<th>Creatine kinase level</th>
<th>125\textsuperscript{i}-Bungarotoxin binding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>arbitrary units</td>
<td>cpm/well</td>
</tr>
<tr>
<td>None</td>
<td>100 ± 11</td>
<td>1351 ± 77</td>
</tr>
<tr>
<td>SNP (30 μM)</td>
<td>97 ± 8</td>
<td>1386 ± 115</td>
</tr>
<tr>
<td>NMMA (100 μM)</td>
<td>95 ± 9</td>
<td>1412 ± 84</td>
</tr>
</tbody>
</table>

TABLE II
Effect of A23187, SNP, or 8-Br-cGMP on reversal of NMMA- and methylene blue-mediated inhibition of myoblast fusion

The cells cultured for 24 h were further cultured in the absence and presence of NMMA or methylene blue (MB) for the next 28 h. They were then treated with A23187, SNP, or 8-Br-cGMP. After incubation for 5 h, extent of myoblast fusion was determined as described under "Experimental Procedures." Similar data were obtained in three independent experiments.

<table>
<thead>
<tr>
<th>Addition (concentration)</th>
<th>Fusion At 24 h of culture</th>
<th>Fusion At 62 h of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
<td>42</td>
</tr>
<tr>
<td>NMMA (0.1 mM)</td>
<td>None</td>
<td>22</td>
</tr>
<tr>
<td>NMMA (0.1 mM)</td>
<td>A23187 (1 μM)</td>
<td>24</td>
</tr>
<tr>
<td>SNP (30 μM)</td>
<td>A23187 (1 μM)</td>
<td>44</td>
</tr>
<tr>
<td>MB (0.6 μM)</td>
<td>A23187 (1 μM)</td>
<td>30</td>
</tr>
<tr>
<td>MB (0.6 μM)</td>
<td>8-Br-cGMP (1 μM)</td>
<td>39</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of NMMA (A) and SNP treatments (B) on cGMP level in cultured myoblasts. A, cells cultured for 24 h were further cultured for various periods in the absence (○) and presence of 0.1 mM NMMA (●). They were then harvested, 50 mM sodium acetate (pH 5.2) preheated to 90 °C was added, and cells were incubated for 1 h at the same temperature. The samples were vigorously vortexed for 2 min, incubated for 30 min on ice, and centrifuged at 13,000 × g for 20 min at 4 °C. The supernatants were acetylated (Harper and Brooker, 1975) and subjected to radioimmunoassay for determination of cGMP concentration (Steiner et al., 1972). The degree of myoblast fusion is also indicated (●). B, cells cultured for 50 h were washed twice with MEM and incubated for 1 h. The medium was replaced with MEM containing 25 mM HEPES (pH 7.4) and 2 mg/ml bovine serum albumin, and the cultures were incubated for 15 min. They were then treated with 0 (○), 10 μM (△), 30 μM (■), 50 μM SNP (●), and 50 μM SNP together with 3 μM methylene blue (†). At the indicated time of incubation, the medium was aspirated off and the cells were immediately frozen on a dry ice/acetone bath. The frozen samples were thawed, centrifuged, and assayed for cGMP as above.
REFERENCES


Cyclic Nucleotide Res. 9, 145–158


