Communication

Nitric Oxide Decreases Cytosolic Free Calcium in Balb/c 3T3 Fibroblasts by a Cyclic GMP-independent Mechanism*

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The purpose of this study was to investigate the effects of NO on cytosolic calcium levels in Balb/c 3T3 fibroblasts that were previously shown to lack soluble guanylate cyclase activity. Authentic NO as well as two NO-generating vasodilators, S-nitroso-N-acetylpenicillamine and isosorbide dinitrate, decreased cytosolic calcium in these fibroblasts. The effect of NO and S-nitroso-N-acetylpenicillamine was concentration-dependent and, for the most part, reversible. Since S-nitroso-N-acetylpenicillamine did not increase either cGMP or cAMP, NO did not increase cGMP, and 8-bromo-cGMP did not alter cytosolic free calcium, we conclude that NO decreases cytosolic free calcium by a cyclic nucleotide-independent mechanism in Balb/c 3T3 fibroblasts.

Recent studies have shown that endothelial cells generate NO via oxidation of L-arginine and that NO (or a closely related substance) is an endothelium-derived relaxing factor (1–3). The release of NO is evident in endothelial cells under basal conditions, and this process is further enhanced by several vasoactive agents (4–6). Endothelium-derived NO, and agents that enhance its release, promote the accumulation of cGMP in various tissues (7, 8) by activation of soluble guanylate cyclase activity, and this action is mimicked by NO-generating vasodilators such as S-nitroso-N-acetylpenicillamine or isosorbide dinitrate (9,10). Many of the biological actions of NO, such as vascular relaxation, decrease of cytosolic free calcium, and inhibition of mitogenesis are also mimicked by 8-bromo-cGMP or atrial natriuretic peptide, a hormone that increases particulate guanylate cyclase activity (11–18). These findings have supported the hypothesis that the effects of NO are mediated by cGMP as the second messenger. However, not all actions of NO appear to be mediated by cGMP, as demonstrated by a recent study indicating that NO induces the ADP-ribosylation of a 39-kDa protein by a cGMP-independent mechanism (19).

According to previous studies, Balb/c 3T3 fibroblasts lack soluble guanylate cyclase activity (20), and these cells may therefore constitute an appropriate experimental model to investigate potential cGMP-independent effects of NO. Cytosolic free calcium is thought to be an important second messenger system in the regulation of a wide variety of cell functions; we have used Balb/c 3T3 cells to determine the effects of NO and NO-generating vasodilators on cytosolic free calcium levels. The results indicate that NO decreases cytosolic free calcium in Balb/c 3T3 cells by a cGMP-independent mechanism.

MATERIALS AND METHODS

Balb/c 3T3 fibroblasts were obtained from the American Type Culture Collection (Rockville, MD) and were used between subcultures 66 and 74. Rat mesangial cells were isolated and cultured as described previously in detail by our laboratory (21, 22). They were used in subcultures 6–11. S-Nitroso-N-acetylpenicillamine (SNAP) was synthesized according to the method of Field et al. (23) by the reaction of NaN3O, and N-acetylpenicillamine at low pH, and characterized as described previously (17). Isosorbide dinitrate and fatty acid-free bovine serum albumin were obtained from Sigma. Insulin, transferrin, and selenium were purchased from Collaborative Research (Bedford, MA), whereas 2'-O-succinyl-1[125]-iodotyrosine methyl ester-guanosine 3',5'-cyclic phosphoric acid or 2'-O-succinyl-1[125]-iodotyrosine methyl ester-adenosine 3',5'-cyclic phosphoric acid (2,200 Ci/ml) were from Du Pont-New England Nuclear. Specific antibody against cGMP and cAMP were purchased from Kew Scientific (Columbus, OH). Dulbecco’s modified essential medium and fetal bovine serum were purchased from Gibco. Fura-2 acetoxyethyl ester was obtained from Molecular Probes (Eugene, OR), and NO gas was obtained from Cryodyne (White Plains, NY).

For experimental purposes, Balb/c 3T3 cells were seeded at a subconfluent density of ~3 × 10⁸ cells/cm² and cultured in medium containing 10% fetal bovine serum, insulin (5 μg/ml), transferrin (5 μg/ml), and selenium acid (5 mg/ml) plus penicillin/streptomycin (100 units/ml and 100 μg/ml, respectively). After 2–4 days in culture, at which time the cells were still subconfluent, they were loaded with fura-2 by incubating them for 1 h at 37 °C with fura-2 acetoxyethyl ester (4 μM) in the presence of 0.1% bovine serum albumin. In some experiments, cells were loaded with fura-2 acetoxyethyl ester in the presence of 0.03% pluronic in addition to 0.1% serum albumin. Since the presence or absence of pluronic did not alter the results overall, most experiments were carried out by loading cells in the absence of pluronic but presence of 0.1% albumin. Intracellular free calcium was measured in fura-2-loaded cells on a Shimadzu model RF6000U fluorescence spectrophotometer in superfused cells, as previously described in detail (24). SNAP, isosorbide dinitrate, or NO did not alter the fluorescence of cells lacking fura-2. Moreover, SNAP (100 μM) did not alter the fluorescence of fura-2 or its association constant with calcium, as demonstrated by the finding that it did not significantly modify the fluorescence ratio of the dye over a wide range of free calcium concentrations (results not shown). Fura-2-loaded cells were superfused using a physiological salt solution of the following composition (in mM): 135 NaCl, 5 KCl, 1 NaHPO₄, 0.5 MgSO₄, 1.8 CaCl₂, 10 glucose, 10 HEPES, 0.025% fatty acid-free bovine serum albumin, pH 7.4.

NO-containing stock solutions were prepared by bubbling NO gas, for 10–15 min, into physiological salt solution that had been previously deaerated by bubbling with nitrogen. NO concentrations in the experimental media were estimated according to a published method (2) by spectrophotometric determination of nitrite ion.

To determine the effect of SNAP on cyclic nucleotide levels, cells were incubated with or without varying concentrations of SNAP and incubations were terminated by aspirating the medium; cyclic nucleotides were extracted with 0.1 N HCl, as described previously (25). The time of incubation for these experiments was 10 min, and this was based on the finding that incubation times ranging from 15 to 10 min gave similar results (results not shown), and therefore a 10-min incubation was routinely used. Cyclic AMP and cGMP levels were determined using specific radioimmunoassay kits.

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†The abbreviations used are: SNAP, S-nitroso-N-acetylpenicillamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
RESULTS

NO and NO-generating Vasodilators Decrease Cytosolic Free Calcium in Balb/c 3T3 Cells—To investigate the effect of NO on cytosolic free calcium, we used two structurally dissimilar NO-generating vasodilators, SNAP and isosorbide dinitrate, as well as authentic NO. As depicted in Fig. 1, SNAP, isosorbide dinitrate, and NO rapidly decreased cytosolic free calcium levels in resting cells. These actions were reversed upon removal of the effectors, except at concentrations of NO greater than 300 μM which also decreased calcium but where washout of NO did not induce a rapid return to basal levels, unlike that found at lower concentrations of NO. It should also be noted that the experiments utilizing NO were done by superfusion of cells with oxygen-lacking medium, in view of the rapid degradation of NO by oxygen. Furthermore, in separate control experiments, we demonstrated that superfusion of Balb/c 3T3 cells with oxygen-lacking medium for up to 30 min did not significantly alter prevailing cytosolic free calcium levels.

To establish that the action of NO was not mediated by one of its degradation products, we exposed NO-containing solutions to air for 30-45 min at room temperature, to allow for the oxygen-mediated decomposition of NO. Following this procedure, we observed that NO degradation products were completely ineffective in decreasing cytosolic free calcium (results not shown), indicating that NO itself rather than a metabolite mediated the decrease of cytosolic free calcium.

Results shown in Fig. 2 indicate that the decrease of cytosolic free calcium elicited by SNAP and NO was concentration-dependent. For both effectors, concentrations in the range of 10-100 μM were found to decrease cytosolic free calcium levels.

8-Bromo-cGMP Does Not Lower Cytosolic Free Calcium in Balb/c 3T3 Fibroblasts—To further investigate the role of
cGMP in mediating the cytosolic calcium-lowering effect of NO, we determined the effect of 8-bromo-cGMP on cytosolic calcium levels in Balb/c 3T3 cells. If the action of NO were to be mediated by cGMP, we would expect 8-bromo-cGMP to mimic this effect. As shown in Fig. 3, 8-bromo-cGMP did not alter calcium levels in fibroblasts, even at high concentrations. In contrast, as expected the cyclic nucleotide significantly decreased cytosolic free calcium in mesangial cells.

Neither SNAP nor Nitric Oxide Increases Cyclic GMP Levels in Balb/c 3T3 Fibroblasts—Previous studies have indicated that Balb/c 3T3 fibroblasts lack the expression of soluble guanylate cyclase activity (20). To verify this aspect, we determined the effect of SNAP on cGMP as well as cAMP accumulation in these cells. Time course experiments revealed that 1 mM SNAP failed to increase cGMP levels in Balb/c 3T3 cells at any time point between 30 s and 30 min (results not shown). Furthermore, as shown in Table I, varying concentrations of SNAP did not significantly alter cGMP levels in either the presence or absence of the cyclic nucleotide phosphodiesterase inhibitor isobutylmethylxanthine (1 mM). In contrast, and as expected, SNAP increased cGMP accumulation in mesangial cells by more than 100-fold (results not shown). Cyclic AMP accumulation in response to SNAP was also determined and found to be invariant (Table I). Similarly, NO (126 or 290 μM) did not increase cGMP levels in cells incubated in deoxygenated medium for 30 s to 2 min (results not shown). These results confirm previous studies that Balb/c 3T3 cells lack the expression of soluble guanylate cyclase activity (20) and provide further support for the notion that NO decreases cytosolic free calcium by a cyclic nucleotide-independent mechanism.

**DISCUSSION**

We and others have previously shown that cGMP and cGMP-elevating agents, including NO, decrease cytosolic free calcium in vascular and renal cells and induce vascular relaxation (13–18). Based on several criteria, including the ability of NO-generating vasodilators to enhance the accumulation of cGMP and of exogenous cGMP analogues to mimic the actions of NO, the assumption of most investigators has been that cGMP mediates the effects of NO. In the current work, we show that NO and vasodilators that generate NO decrease cytosolic free calcium in Balb/c 3T3 fibroblasts by a cGMP-independent mechanism. This conclusion is based on these findings: 1) Two structurally dissimilar NO-generating vasodilators as well as authentic NO decrease cytosolic free calcium levels. The decrease elicited by NO or SNAP is dose-dependent. 2) SNAP which can increase cGMP levels in mesangial cells by more than 100-fold has no effect on either cGMP or cAMP levels in Balb/c 3T3 fibroblasts; similarly, NO fails to increase cGMP levels in these cells. 3) 8-Bromo-cGMP decreases cytosolic calcium in mesangial or smooth muscle cells but does not alter cytosolic free calcium levels in fibroblasts. The Balb/c 3T3 fibroblasts therefore seem to lack not only soluble guanylate cyclase activity but responsiveness to exogenous cGMP analogues as well.

Cyclic AMP has also been shown to alter cytosolic free calcium levels in various tissues (27). It was therefore of interest to investigate whether SNAP altered cAMP levels in Balb/c 3T3 fibroblasts. As indicated in Table I, cAMP levels did not change in the presence of SNAP. Thus, the effects of NO cannot be attributed to altered cAMP levels.

The recent findings of Brüne and Lapetina (19), demonstrating that NO induces ADP-ribosylation of a 39-kDa protein in platelets and several other tissues, may be relevant to our findings because this effect was also shown to occur by a cGMP-independent mechanism. Whether NO induces ADP-ribosylation in Balb/c 3T3 fibroblasts and whether the decrease of cytosolic free calcium and the stimulation of ADP-ribosyltransferase activity are related is a question that will require additional work.

In summary, our results support the concept that NO influences cell function by a cGMP-independent mechanism, based on the observation that NO modulates cytosolic free calcium in Balb/c 3T3 fibroblasts in which both the expression of soluble guanylate cyclase activity and responsiveness to cGMP are lacking.

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