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# INTERLEUKIN-1 ß INDUCES THE PRODUCTION OF AN L-ARGININE-DERIVED RELAXING FACTOR FROM CULTURED SMOOTH MUSCLE CELLS FROM RAT AORTA

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SUMMARY: The effect of interleukin-1 ß on the production of non-prostanoid vasoactive factors by cultured rat aortic smooth muscle cells was investigated. Under bioassay conditions, the perfusate from a column of confluent cells grown on beads and treated with interleukin-1 ß (1 ng/ml for 18 to 24 hr) abolished the contraction of a canine coronary ring without endothelium contracted by phenylephrine (1 µM), while the perfusate from control cells had no effect. The relaxing activity of the perfusate was observed when transit times were increased from 1 sec to 5 min. Nitro L-arginine (100 µM) reversed the relaxations and L-arginine stereoselectively restored the relaxations. Interleukin-1 ß (1 ng/ml) evoked a time-dependent accumulation of cyclic GMP but not cyclic AMP in cultured smooth muscle cells. The transfer of fresh or stored (-70°C) conditioned culture medium from interleukin-1 ß-treated cells but not from control cells, to cultured smooth muscle cells stimulated the production of cyclic GMP. These observations demonstrate that interleukin-1 ß induces the production of transferable factor which relaxes vascular smooth muscle and stimulates the production of cyclic GMP. 

The conversion of L-arginine to the short-lived free radical nitric oxide and L-citrulline is an ubiquitous biochemical pathway that has been identified for example, in activated macrophages, endothelial cells and neutrophils (1-3). A constitutive and calcium-sensitive nitric oxide synthase is present in endothelial cells and also in cerebellar cells while a calcium-insensitive enzyme is inducible in macrophages and related cell lines by cytokines (4-6). The production of nitric oxide participates in the

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regulatory role of the endothelial cells on the tone of the vasculature and also in the cytostatic activity of macrophages against tumor cells (2,7).

Lipopolysaccharide, a constituent of gram negative bacteria cell walls, as well as cytokines such as interleukin-1 ß, impair the contractility of isolated vascular preparations in an endothelium-independent fashion (8-13). L-arginine the substrate for the production of nitric oxide, evokes concentration-dependent relaxations in rings of bovine pulmonary artery and rat aorta without endothelium which have been incubated for several hours in physiological solution (14,15). These relaxations are inhibited by competitive inhibitors of nitric oxide synthase and also by inhibitors of soluble guanylate cyclase (10-15). These observations suggest that an L-arginine-nitric oxide-like pathway is present in the vessel wall outside of the endothelium although the cell type responsible has not been identified. The present study was designed to investigate whether or not interleukin-1 ß stimulates the production of a relaxing factor by inducing an L-arginine-nitric oxide like pathway in cultured rat aortic smooth muscle cells.

## MATERIALS AND METHODS

### Materials

Acetylcholine, phenylephrine, indomethacin, elastase (EC 3.4.21.4), collagenase (EC 3.4.24.3) and monoclonal antibodies against smooth muscle α-actin were obtained from Sigma Chemical Co. (St Louis, MO); interleukin-1 ß from Boehringer Mannheim Biochemicals (Indianapolis, IN); nitro L-arginine from Aldrich Chemical Co. (Milwaukee, WIS) and reagents for cell culture from Whittaker (Walkesville, MD). All plastic ware was from Costar Corporation (Cambridge, MA) and cytodex 3 beads from Pharmacia (Uppsala, Sweden).

### Cell culture

Vascular smooth muscle cells were isolated by elastase and collagenase digestions of thoracic aortas from Wistar rats, propagated and characterized by immunocytochemical techniques using specific monoclonal antibodies against α-smooth muscle actin as described (16). Cells (from passage 1 to 19) were seeded into either 6 well multiwell plates or T35 flasks containing suspensions of cytodex 3 beads in minimal essential medium (MEM) containing 2 mM glutamine, 20 mM TES-NaOH, 20 mM HEPES NaOH (both pH 7.3), 10% heat-inactivated serum, and 100 U/ml each of streptomycin and penicillin. When cells reached confluence, the culture medium was changed with serum-free MEM for 24 hr to render them quiescent and they were then exposed to interleukin-1 ß in medium containing 1% plasma derived serum (16).

### Bioassay experiments

The left circumflex coronary artery was removed from mongrel dogs anesthetized with sodium pentobarbital (30 mg/Kg i.v.) and placed in a

physiological salt solution (composition in mM: NaCl, 118.3; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $KH_2PO_4$, 1.2; $NaHCO_3$, 25.0; calcium edetate, 0.026 and glucose, 11.1). The arteries were cleaned of fat and connective tissue and cut into rings (3 mm long). After mechanical removal of the endothelium, the rings were mounted between a stirrup anchored to a steel plate and one connected to a strain gauge transducer (FT03D, Grass Instrument Co., Quincy, Mass., USA) for recording of changes of isometric tension. The detector rings were superfused with the perfusate from either columns containing beads without cells (direct superfusion) or columns containing beads covered with smooth muscle cells. The columns of beads were perfused at constant flow (3 ml/min) with physiological salt solution at 37°C and bubbled with a 95% $O_2$ plus 5% $CO_2$ gas mixture. The detector rings were progressively stretched to the optimal point of their length-active tension curve (7 to 9 g). The absence of endothelium was confirmed by the lack of relaxation evoked by acetylcholine (1 μM) on the detector ring contracted by phenylephrine (1 μM) under direct superfusion. Columns containing cells were perfused for 45 min before the beginning of experiments. All experiments were performed in the presence of indomethacin (10 μM) in order to prevent the production of vasoactive prostanoids.

Measurement of cyclic GMP

Indomethacin (10 μM) and IBMX (100 μM; a non-selective inhibitor of phosphodiesterases) were added to confluent cultures of smooth muscle cells in 35 mm Petri dishes, 45 min before the addition of interleukin-1 ß or conditioned medium. After incubation, medium was removed by aspiration, cells scraped off in trichloroacetic acid (6%) and collected into an Eppendorf tube. Cell suspensions were sonicated for 5 sec before centrifugation for 10 min at 13,600xg. Supernatants were extracted with four volumes of water-saturated ether and then lyophilized. The content of cyclic GMP of each sample was measured by using a radioimmunoassay kit (Biomedical Technologies Inc., Stoughton, MA). All experiments were performed on triplicated wells.

Statistical analysis

Results are expressed as means ± S.E.M.; n represents the number of coronary arteries in bioassay experiments and the number of wells studied in the measurement of cyclic GMP levels. Statistical comparisons were made by using Student's paired t-test. P values less than 0.05 were considered to be statistically significant.

## RESULTS

Bioassay experiments

The effluent of rat aortic smooth muscle cells incubated in culture medium containing interleukin-1 ß (1 ng/ml for 18 to 24 hrs) prior to superfusion rapidly inhibited the contraction evoked by phenylephrine (1 μM) as well as the myogenic tone of coronary rings without endothelium (Fig. 1A,B; transit time: 1 sec). Whereas effluent from control cells had no

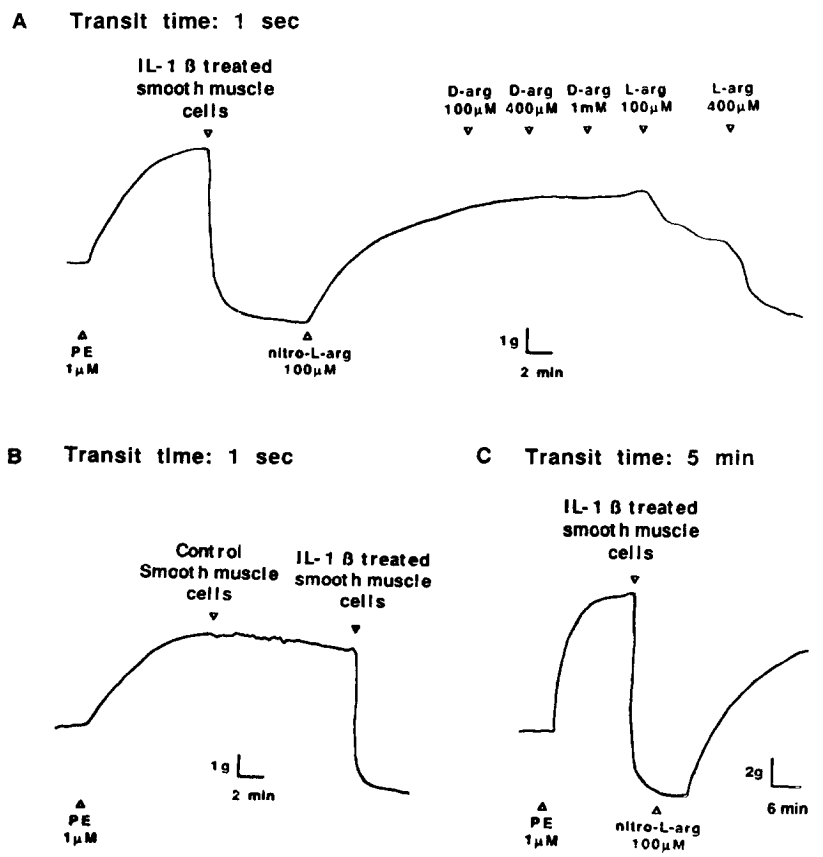


Fig. 1. (A) Bioassay experiments demonstrating the relaxing activity of the perfusate, from a column of cultured rat aortic smooth muscle cells grown on microcarrier beads and treated with interleukin-1 ß (IL-1 ß, 1 ng/ml) for 24 hr, on a canine coronary artery ring without endothelium contracted with phenylephrine (PE, 1 µM). Nitro L-arginine (100 µM) inhibited the relaxation and L-arginine but not D-arginine restored the relaxation. (B) The effluent of a column of control cultured rat aortic smooth muscle cells did not affect the contraction evoked by phenylephrine. (C) A similar relaxing activity of the perfusate was detected when the transit time between the donor cell column and the detector was increased from 1 sec to 5 min. Similar observations were made with five different isolates of smooth muscle cells (2.6 ± 0.3 million cells per column). Experiments were performed in the presence of indomethacin (10 µM).

such effect (Fig. 1B). The relaxations were sustained and were detected after 5 hrs of perfusion of columns containing cells with physiological salt solution (data not shown). A similar relaxation was obtained when the transit time between the cell column and the detector ring was increased to 5 min (Fig. 1C). The relaxations evoked by the perfusate were reversed by infusion of nitro L-arginine (100 µM) over the column of cells (Fig. 1A) but not over the detector bioassay ring (data not shown). The relaxations

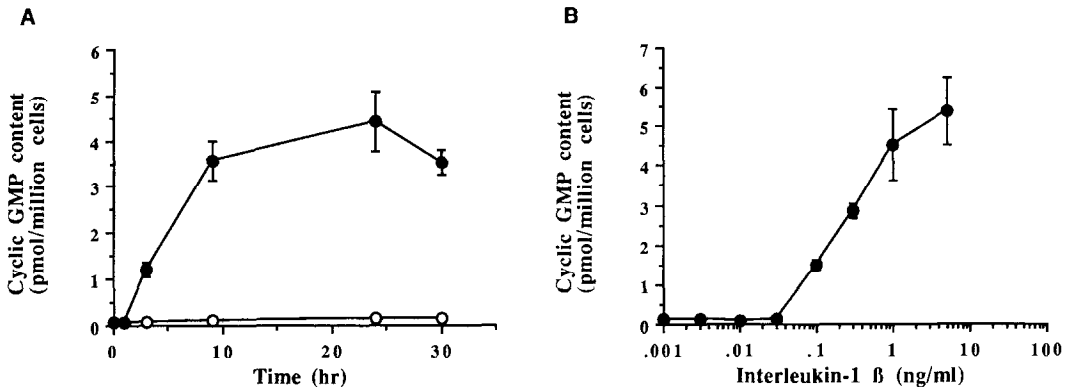


Fig. 2. (A) Kinetics of the production of cyclic GMP in control (open circles) and interleukin-1 ß (1 ng/ml; full circles)-treated rat aortic cultured smooth muscle cells. (B) Production of cyclic GMP evoked by a 24 hr exposure of cultured smooth muscle cells to increasing concentrations of interleukin-1 ß. The experiments were performed in the presence of IBMX (100 µM) and Indomethacin (10 µM). Results are shown as means ± S.E.M. of three different experiments.

were restored by infusion of L-arginine but not D-arginine over the column of cells (Fig. 1A).

Production of cyclic GMP

The content of cyclic GMP remained unchanged in confluent cultures of smooth muscle cells during the first hour of treatment with interleukin-1 ß (1 ng/ml). Thereafter, a time-dependent accumulation of cyclic GMP but not of cyclic AMP (data not shown), was induced by cytokine-treatment and this reached a maximum after 9 hrs and remained stable for the next 21 hrs (Fig. 2A). The production of cyclic GMP was concentration-dependent (Fig. 2B). The addition of conditioned medium from interleukin-1 ß-treated cells (1 ng/ml; 24 to 30 hr) but not from control cells, to smooth muscle cells for two min produced an accumulation of cyclic GMP (Fig. 3A). This stimulated production of cyclic GMP by conditioned medium from interleukin-1 treated cells was observed even after its storage at -70°C for 5 to 30 days (Fig. 3B).

## DISCUSSION

The results show that treatment of cultured rat aortic smooth muscle cells with interleukin-1 ß caused the release of a transferable factor that relaxes isolated blood vessels and stimulates the production of cyclic GMP.

The relaxing factor produced by cells treated with interleukin-1 ß was not due to normal constituents of the cell culture medium since they were removed by the extensive washing (45 min) of cells with physiological salt solution prior to performance of bioassay experiments.

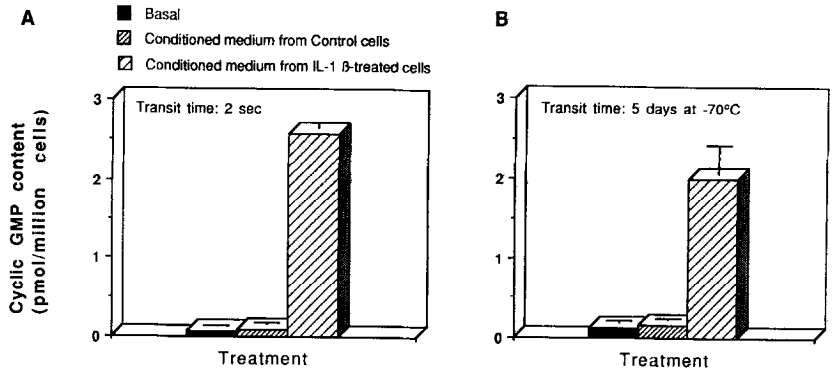


Fig. 3. Effects of conditioned medium collected after a 30 hr incubation period of control or interleukin-1 ß (1 ng/ml)-treated smooth muscle cells on the production of cyclic GMP in different smooth muscle cell cultures. The conditioned medium was transferred either (A) directly (2 sec delay) or (B) after storage at -70°C for 5 days. The experiments were performed in the presence of IBMX (100 µM) and indomethacin (10 µM). Results are shown as means ± S.E.M. of a representative experiment performed on three wells. Similar observations were made in two additional experiments (2 sec delay) and also with conditioned medium stored at -70° C for 6, 10 and 28 days.

The release of the relaxing factor persisted for up to five hours of continuous perfusion of the cells with physiological salt solution. This observation, in conjunction with the delayed onset of the production of cyclic GMP and the fact that treatment of the cells with cycloheximide abolished the vasorelaxant activity of the perfusate (17), suggest that protein synthesis is a prerequisite in the production of the relaxing factor by interleukin-1 ß.

Treatment of columns of cells with nitro L-arginine (a competitive inhibitor of nitric oxide synthase; 18,19) reversed the relaxations evoked by the perfusate; this effect was overcome stereoselectively by L-arginine. The relaxing activity of the perfusate is inhibited also by methylene blue (an inhibitor of soluble guanylate cyclase, 17). Interleukin-1 ß stimulates an intracellular accumulation of cyclic GMP but not of cyclic AMP. Altogether, these observations favor the hypothesis that interleukin-1 ß stimulates the production of a nitric oxide-like factor in cultured smooth muscle by inducing nitric oxide synthase.

These results identify smooth muscle cells as a likely candidate for the production of a nitric oxide-like factor in the vessel wall which mediates, at least in part, the impaired endothelium-independent contraction of isolated vascular preparations incubated for several hours with interleukin-1 or other cytokines (8-13). They are consistent also with the claims that cytosol from cytokine-treated smooth muscle cells activate soluble guanylate cyclase (12).

The relaxing activity of the perfusate from columns of cells treated with interleukin-1 ß was not affected by increasing the length of the tube between the donor cell column and the detector ring from 1 sec to 5 min. Conditioned medium from interleukin-1 ß-treated cells induced a production of cyclic GMP even after storage at -70°C for several days. These observations suggest that the relaxing factor produced by interleukin-1 ß-treated smooth muscle cells may be different to endothelium-derived nitric oxide which biological half-life ranges from 6 to 50 seconds (20,21). Alternatively, interleukin-1 ß may stimulate a large production of a nitric oxide-like factor in smooth muscle cells. It is possible that after a transit time of 5 min or following storage of the conditioned medium at -70°C, the radical may still be present in the perfusate and conditioned medium in amounts sufficient to evoke full relaxation of the detector ring and evoked a production of cyclic GMP, respectively. This view is supported by the observation that a solution of authentic nitric oxide (1%) was able to evoke small relaxations (20 to 25% of its initial response) in rat aortic rings without endothelium following its storage at -70°C for seven days (Junquero and Schini, unpublished observations). A similar relaxing factor which can be stored at -80°C for seven days, and which is synthesized de novo from L-arginine is obtained also from rat peritoneal neutrophils (23). The identity of this relaxing factor is unknown however it is tempting to speculate that it may be an endogenous donor of nitric oxide which plays an important role in vascular homeostasis.

## ACKNOWLEDGMENTS

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