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MECHANISM FOR THE GENERATION OF ACTIVE SMOOTH MUSCLE INHIBITORY FACTOR (IF) FROM BOVINE RETRACTOR PENIS MUSCLE (BRP)

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Summary The active smooth muscle inhibitory factor (IF) is generated by acidifying the extracts from the bovine retractor penis muscle (BRP). The activated IF is very similar to endothelium-derived relaxing factor (EDRF). However, the mechanism for the generation of active IF is yet unknown. From reverse-phase high performance liquid chromatographic data, the activities of nitrite loss were closely related to the contents of both sulfhydryl (SH) groups and L-cysteine. Thus, under acidic conditions, RSH and nitrite in the extracts are considered to react as follows: $RSH + HNO_2 \rightarrow RSNO + H_2O$, $RSNO \rightarrow (1/2)RSSR + NO$, $NO + (1/2)O_2 \rightarrow NO_2$, $NO_2 + (1/2)H_2O \rightarrow (1/2)HNO_2 + (1/2)HNO_3$. In addition to NO, RSNO is also a labile but potent vasodilator.

Thus, activated IF seems to consist of RSNO and NO, and thiol-containing small molecules (molecular weight of less than 1,000) from BRP are closely involved in the generation of active IF.

Recently, evidence has been presented that endothelium-derived relaxing factor (EDRF) is nitric oxide (NO) (1,2). Smooth muscle inhibitory factor (IF) extracted from the bovine retractor penis muscle (BRP) is another smooth muscle relaxing agent (3-5). There are several similarities between IF and EDRF (5-7).

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Abbreviations used in this paper: IF, inhibitory factor; BRP, bovine retractor penis muscle; EDRF, endothelium-derived relaxing factor; SH, sulfhydryl; NDRF, neutrophil-derived relaxing factor.

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Martin et al. reported that extracts of BRP contain nitrite, and acid-activated IF relaxes smooth muscle and is associated with the loss of nitrite. They considered that activated IF is NO liberated from NO_2^- when the extracts are acidified (5). When sodium nitrite was acidified, only a weak and transient vasodilator activity was observed. Thus, BRP extracts were suspected to contain an unknown NO-stabilizing agent that promotes the conversion of nitrate to NO.

In this paper, we found that sulfhydryl (SH) groups are involved in the generation of active IF.

Materials and Methods

Preparation and purification of inhibitory factor (IF) from bovine retractor penis (BRP)

Preparation of BRP (400g) and the purification of IF by anion exchange column and activated alumina column were performed according to the method by Martin et al (5). All procedures except for the high-performance liquid chromatographic separation were performed at 4°C. Eluates (10 ml) from the alumina column was passed through a Diaflo Ultrafiltration membrane 5 YCO 5 (molecular cut off level as low as 1,000; Amicon, MA, USA). All activities were recovered in the filtrate. One half milliliter of the filtrate was injected into a high-performance liquid chromatographic system (System Gold, Beckman Instruments, CA, USA). The column was YMC A-314, ODS (C18)(0.6 cm x 40 cm, Yamamura Chemical Company, Kyoto, Japan) (Figure 1-A).

Nitrite determination by diazo formation

The loss of nitrite was measured by diazo formation (8). At room temperature, 0.5 ml of a 1 to 10 diluted sample from each fraction was mixed with 0.1 ml of 10mM $NaNO_2$ and 0.1 ml of 1 N HCl. After one hour, 0.25 ml of 1% (w/v) sulfanilic acid in 2 N HCl was added. After five minutes, 0.25 ml of 1% (w/v) aqueous N-(1-naphthyl) ethylenediamine dihydrochloride was mixed in, and the absorbance was determined at 548 nm (Figure 1-B).

Assay of sulfhydryl (SH) groups

The determination of SH groups was performed according to the method by Ellman (9). Fifty microliters of sample was mixed with 940 µl of 0.02 M EDTA in 0.2 M Tris.HCl (pH 8.3) and 10 µl of 10 mM DTNB (5,5'-dithiobis(2-nitrobenzoic acid)) . After 10 minutes, the absorbance was assayed at 412 nm. Calibration was obtained using L-cysteine (Figure 1-C).

L-cysteine determination in each fraction

L-cysteine determination was performed as follows (10,11): 0.2 ml of sample was mixed with 0.1 ml of performic acid at 0°C. After 1.5 hr, 0.05 ml of 48% hydrobromic acid was added, and the sample was dried in vacuo. After adding 0.2 ml of 6 N HCl, 24 hr hydrolysis was performed at 110°C. After hydrolysis, 300 µl of 0.1 N HCl was added. Twenty microliters of solution was mixed

with 20 µl of PITC (phenylisothiocyanate) solution (v/v, ethanol:triethylamine:H_2O:PITC=7:1:1:1). After 20 minutes, it was dried in vacuo. One hundred microliters of 5% acetonitrile-5 mM phosphate buffer (pH 7.4) was added and 5 µl aliquots were injected into the HPLC. HPLC analysis was performed by the Pico-Tag system (Waters, MA, USA). Solvent A: 0.14 M NaOAc, 0.2% ET_3/CH_3CN(95:5), Solvent B: CH_3CN/H_2O(60:40). Gradient conditions: 0 min, 100% A, 4 min, 90% A, and 15 min, 60% A. UV detection was performed at 254 nm.

Tension Recording

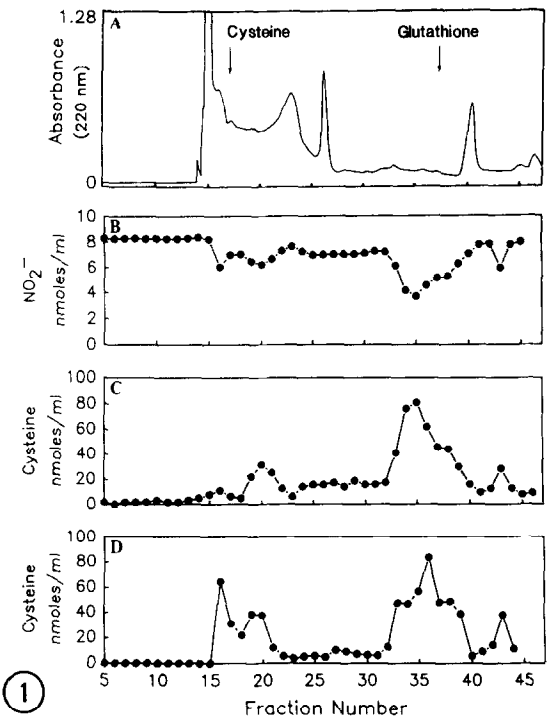
Tension recording was performed at 37°C as previously described (12,13). The modified Krebs solution of the following composition was used: 121.9 mM NaCl, 4.7 mM KCl, 2.54 mM $CaCl_2.2H_2O$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 15.5 mM $NaHCO_3$, and 11.5 mM glucose, pH 7.5.

Results and Discussion

Figure 1-A is a chromatogram at 220 nm obtained by RP-HPLC. Figure 1-B shows the activities of losing NO_2^-, i.e., vasodilating activity. The relation between NO_2^- concentration (nmoles/ml, Y axis) and vessel relaxation (%, X axis) is as follows: Y=8.3-0.1 X, r=0.91, $P<0.001$. Figures 1-C and 1-D show the sulfhydryl (SH) contents calculated as L-cysteine equivalent and the L-cysteine concentration determined by the amino acid analysis after the hydrolysis of each fraction. From this experiment, it was found that the activity of losing NO_2^- corresponded closely to the SH content and L-cysteine concentration. In fraction No.16 in Figure 1-D, the relatively high concentration of cysteine compared with Figures 1-B and 1-C was probably due to the increased absorption in the void fraction of RP-HPLC.

The molecular weight of IF seemed to be just under 1,000 as judged by its ability to pass through the ultrafiltration membrane. The recovery rate of activity in RP-HPLC was 90%.

From the above results, cysteine or cysteine containing small molecules (RSH) eluted by methanol seemed to be responsible for the activation of IF extracted from BRP. The eluted positions of cysteine and glutathione (l-glutamyl-l-cysteinyl-l-glycine) are also shown in Figure 1-A.



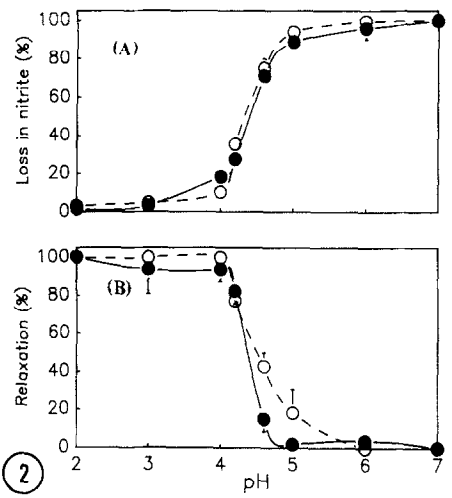


Fig.1: Analysis of each fraction by RP-HPLC after anion exchange and alumina chromatography.
(A) The carrier was 0.024 N HCl with a flow rate of 1 ml/min. UV monitoring was performed at 220 nm. The effluent was collected in 0.5 ml fractions. Finally, the column was washed with 100% methanol, but no peaks appeared.
(B) activity of losing NO_2^-.
(C) SH content expressed as L-cysteine concentration.
(D) L-cysteine determination of each fraction from RP-HPLC.

Fig.2: Effect of pH on nitrite loss and vessel relaxation in the presence of cysteine (closed circle),and glutathione (open circle).
Buffers employed in this experiment were 50 mM glycine-HCl (pH 2.0 and 3.0), 50 mM sodium acetate (pH 4.0, 4.2, 4.6 and 5.0) and 50 mM sodium phosphate (pH 6.0 and 7.0). After incubating one ml of the reaction mixture (15 µl of 15 µM $NaNO_2$, 10 mM cysteine or 11.5 mM glutathione, 500 µl of each buffer, and 385 µl of H_2O) for 15 minutes at 37°C, one ml of 1% (w/v) sulfanilic acid in 2 N HCl and one ml of 1%(w/v) N-(1-naphtyl)ethylenediamine chloride were added (Fig.2-A, n=5). For the relaxation experiment, after 15 minutes, a 5 µl aliquot from each tube at 37°C was added to the bath (Fig.2-B, n=5).

Figure 2 shows the effect of cysteine and glutathione on the loss of nitrite and relaxing activities. Both phenomena occur rapidly below pH 5.0.

The three smooth muscle relaxing factors, endothelium-derived relaxing factor (EDRF) (1,2), neutrophil-derived relaxing factor (NDRF) (15,16) and active inhibitory factor (IF) derived from bovine retractor penis (BRP) (3-7), have very similar phar-

macological and chemical properties: They are labile, anionic and hydrophilic; They act by stimulating guanylate cyclase; and their activities are blocked by hemoglobin. NO has been proposed to be the common candidate for these three factors. Active IF from BRP is generated by adding HCl to the extracts of BRP. This extract has nitrite. However, in vitro, the combination of nitrite and HCl does not produce a relaxing agent or NO. Thus, in addition to nitrite and HCl, other factors may be necessary for producing active IF.

In this paper, we disclosed the mechanism for the generation of active IF, which has been unknown for the past 10 years. By RP-HPLC, we found that the activity of losing NO_2^- (i.e., relaxing activity) is closely related to the content of SH groups. In the presence of RSH, the following reactions (A)-(D) were considered to occur:

(A) $RSH + HNO_2 \longrightarrow RSNO + H_2O$, (B) $RSNO \longrightarrow (1/2)\ RSSR + NO$

(C) $NO + (1/2)\ O_2 \longrightarrow NO_2$, (D) $NO_2 + (1/2)\ H_2O \longrightarrow$

$(1/2)\ HNO_2 + (1/2)\ HNO_3$

RSNO is a labile but potent vasodilator (14). Thus, the activated IF is considered to be composed of RSNO and NO.

Recently, it has been reported that EDRF and NO are not identical (17). One of the technical problems in detecting NO is that the chemiluminescence technique coupled with acidification preprocessing nonspecifically cleaves NO from several nitroso compounds. This raises the possibility that the detected NO may be derived from another compound. Thus, EDRF may be a nitroso compound that releases NO by this technique.

In BRP, the origin of NO_2^- is unknown. NO may also be produced from L-arginine like endothelial cells in culture (2), and through reactions (C) and (D), NO may be changed to NO_2^- and NO_3^-.

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